

## The Expression and Activity of D-Type Cyclins in F9 Embryonal Carcinoma Cells: Modulation of Growth by RXR-Selective Retinoids

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### INTRODUCTION

The growth rate of malignant F9 embryonal carcinoma cells slows considerably following all-trans-retinoic acid-induced differentiation into benign parietal endoderm. To determine the mechanism of this process, we examined the expression of cyclins D1, D2, and D3 and the activity of their associated kinases. Cyclin D1 and D3 mRNA levels decreased during complete differentiation induced by all-trans-retinoic acid and dibutyryl cAMP, while the levels of cyclin D2 and the cyclin-dependent kinase (Cdk) inhibitor p27 mRNAs increased. Ultimately, terminally differentiated cells possessed 50% of the Cdk4-associated kinase activity observed in undifferentiated cells. Since numerous genes are differentially regulated during parietal endoderm differentiation, it is difficult to determine whether retinoic acid affects cell cycle gene expression directly or if these changes are caused by differentiation. We found that the retinoid X receptor (RXR)-selective agonists LG100153 and LG100268 significantly inhibited F9 cell growth without causing overt terminal differentiation as assessed by anchorage-independent growth and differentiation-associated gene expression. As seen in cells induced to differentiate by the RAR agonist all-trans-retinoic acid, RXR activation led to an increase in the number of cells in G1 phase. RXR agonists also sharply induced the levels of the Cdk regulatory subunits, cyclin D2 and D3. However, Cdk4-dependent kinase activity was reduced by RXR-selective retinoid treatment. These observations suggest that some retinoids can directly inhibit proliferation and regulate Cdk4-dependent kinase activity without inducing terminal differentiation. © 1999 Academic Press

**Key Words:** retinoids; RXR; F9 embryonal carcinoma; differentiation; cyclins.

Vitamin A and its chemical relatives, the retinoids, are important regulators of cell proliferation and differentiation in a diverse array of tissues. Cell types whose *in vitro* differentiation is influenced by retinoids include keratinocytes, chondrocytes, adipocytes, hematopoietic cells, and numerous neoplastic cell lines [1, 2]. Retinoids slow or arrest the growth of many transformed cell lines by inducing differentiation. This observation contributed to the development of a novel chemotherapeutic approach known as differentiation therapy. Rather than selectively killing tumor cells, tumor cells are induced to differentiate. These nonproliferating cells often lose the markers characteristic of the malignant state and gain markers of terminal differentiation.

One promising differentiation agent is the vitamin A derivative retinoic acid (RA). RA reverses the malignancy of several tumor lines *in vitro* and represses acute promyelocytic leukemia and aerodigestive tract tumors [3]. *In vitro*, and presumably *in vivo*, RA exerts its effect by activating a cascade of differential gene expression ending in terminal differentiation and the loss of malignancy.

RA-regulated gene expression is mediated by nuclear retinoid receptors which act as ligand-dependent transcription factors [4]. These receptors are encoded by six different genes, the RARs  $\alpha$ ,  $\beta$ , and  $\gamma$  and the RXRs  $\alpha$ ,  $\beta$ , and  $\gamma$ . Numerous isoforms arising from differential promoter usage and alternate splicing have been identified and the expression of these isoforms is developmentally regulated [5]. Furthermore, the receptors can act as homodimers and heterodimers, each with unique characteristics [see 6, 7]. While the RARs bind to and are activated by all-trans-RA and 9-cis-RA, the RXRs are activated only by 9-cis-RA. The RXRs form heterodimers with the RARs and several other receptors, including the thyroid hormone, vitamin D, oxysterol, peroxisome proliferator-activated receptors, and Nurr77/NGFI-B [8-10]. This plethora of receptors and gene pathways may begin to explain the multiple effects retinoids have on differentiation.

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Unfortunately, toxic side effects limit the clinical use of retinoids. For example, retinoids are extremely teratogenic. Accutane (13-cis-RA), used for treatment of chronic cystic acne, is one of the most potent human teratogens. Babies who survive to birth exhibit malformations of the cardiovascular system, face, and central nervous system and absence of the thymus [11]. The pleiotropic effects of natural retinoids indicate that multiple mechanisms contribute to these detrimental outcomes. To ameliorate the toxicity and teratogenicity of retinoids, receptor-selective agonists have been synthesized which possess a narrower spectrum of activity [12]. These retinoids have clinical and research value, as they affect the expression of a subset of the target genes (e.g., *Bmp-2* and *Bmp-4*) regulated during RA-induced differentiation [13]. Understanding the effects of these retinoids will aid in designing more effective and safe therapeutic agents.

F9 embryonal carcinoma cells are a classic model system for studying the mechanisms underlying RA-induced terminal differentiation. F9 cells can be induced by RA and drugs that increase cellular cAMP levels to differentiate into a pure population of parietal endoderm-like cells [14]. This differentiation is characterized by a change in morphology to that typical of parietal endoderm and the induction of numerous parietal endoderm genes. This synchronous and reproducible differentiation is ideal for biochemical analyses of retinoid-induced responses. As occurs during the terminal differentiation of many cell types, F9 cells cease growing rapidly and lose their malignant phenotype, including their ability to grow in the absence of substrate. The decreased growth rate could be purely the result of terminal differentiation or retinoids could directly inhibit the cell cycle system controlling growth rate. Most likely, a combination of these mechanisms plays a role.

To understand the mechanism that lengthens the F9 G1 phase during terminal differentiation, we have analyzed the expression and activity of several G1 phase regulatory proteins. Cyclins D1, D2, and D3 are generally considered to be positive regulatory subunits of the cyclin-dependent kinase 4 (Cdk4) and Cdk6 kinases. As such, increased cyclin D levels promote G1 progression in several cell types [15]. The G1 Cdks are also regulated by the Cdk inhibitors p21<sup>WAF1/CIP1/CDKN1</sup>, p27<sup>Kip1</sup>, p16<sup>INK4A/MTS1/CDKN2A</sup>, and p15<sup>INK4B/MTS2</sup>. For a general review, see Ref. [16]. We show here that cyclins D1 and D3 levels are reduced and cyclin D2 and p27<sup>Kip1</sup> levels are increased in terminally differentiated parietal endoderm cells relative to undifferentiated F9 stem cells. It was not clear, however, whether these changes required terminal differentiation or whether all-trans-RA directly influenced the expression of these genes.

It has been reported that untreated F9 cells lacking a functional RXR gene grow more rapidly than wild-

type cells [17]. We now report the complementary observation. Specifically, RXR-activating retinoids decrease the rate of F9 cell growth. We also show that RXR activation alters the expression of distinct cell cycle proteins in the absence of RAR-activating retinoids. This occurs without complete terminal parietal endoderm differentiation, suggesting that retinoids can directly influence the cell cycle apparatus.

## MATERIALS AND METHODS

**Cell proliferation and differentiation.** All-trans-RA (RAR agonist), dibutyryl cAMP, theophylline, calf serum, L-glutamine, and  $\beta$ -mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). 9-cis-RA (RAR and RXR panagonist) and TTNPB (RAR agonist) were obtained from Hoffman-LaRoche (Nutley, NJ). LG100153 (LG153, RXR agonist) [18] and LG100268 (LG268, RXR agonist) [19] were obtained from Ligand Pharmaceuticals (San Diego, CA). Stocks (1 mM) of retinoid solutions were prepared in ethanol and diluted to the appropriate concentration into media before use. Monolayer F9 or RA-51 embryonal carcinoma cells [20] were maintained in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, and 0.1 mM  $\beta$ -mercaptoethanol on gelatinized tissue culture plates in 10% CO<sub>2</sub> at 37°C. For differentiation, monolayers were dispersed into single cells with trypsin-EDTA (Gibco-BRL) and replated at the desired density. After cell attachment, the medium was replaced with medium containing the desired concentration of retinoid plus 250  $\mu$ M dbcAMP and 500  $\mu$ M theophylline (CT). On the third day of long experiments, the medium was replaced with fresh medium containing drugs.

**Cell proliferation assays.** F9 cells ( $4.5 \times 10^5$ ) were plated in 0.15 ml DMEM in 96-well flat-bottomed culture plates. After cells attached, the medium was replaced with drug-containing medium. For the MTS assay, separate stock solutions of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulophenyl)-2H-tetrazolium, inert salt) and phenazine methosulfate (PMS) were prepared and added to the cultures to a final concentration of 100  $\mu$ M MTS and 5  $\mu$ M PMS. The amount of formazan product was assayed by measuring the absorbance at 570 nm [21]. For toluidine blue staining, cells grown similarly in 96-well plates were fixed with 0.1 ml of 4% paraformaldehyde for 30 min and then 0.05 ml 0.5% toluidine blue was added and incubated 1 h. The cells were solubilized with 0.1 ml 2% SDS per well and the absorbance was read at 650 nm [22]. For cell counting assays,  $3 \times 10^4$  cells were plated in 1.0 ml medium in 24-well plates. Drugs were added as described above. After 3 or 4 days, cells were trypsinized and counted on a hemacytometer.

**Flow cytometry.** Cells were dispersed with trypsin-EDTA and then fixed with 70% ethanol at -20°C for at least 30 min. The fixed cells were washed with PBS and then resuspended in PBS containing 100  $\mu$ g/ml RNaseA and 40  $\mu$ g/ml propidium iodide. Cells were incubated at 37°C for 30 min. Samples were analyzed for DNA content on a Becton Dickinson FACSCAN using MODFIT software.

**Anchorage-independent growth.** The anchorage independent growth assay was adapted from Chen et al. [23]. The bottom agar layer was prepared by mixing equal volumes of sterilized 1.4% agar (Difco, Sparks, MD) and 2 $\times$  DMEM supplemented with 20% heat-inactivated calf serum, 4 mM L-glutamine, and 0.2 mM  $\beta$ -mercaptoethanol. A quantity of 3.0 ml was pipetted into 60-mm non-tissue culture petri dishes and incubated at 37°C in 10% CO<sub>2</sub> overnight. A cell slurry was prepared by mixing one part 1.4% agar at 45°C, two parts cell suspension ( $1.3 \times 10^6$  cells/ml suspended in 1 $\times$  DMEM), and one part 2 $\times$  DMEM. The cell slurry also contained 250  $\mu$ M dbcAMP, 500  $\mu$ M theophylline, and 0.1  $\mu$ M retinoid or ethanol vehi-

TABLE 1

Percentage of F9 Cells in G1, S, and G2/M Phases Following All-trans-RACT and LG153 CT Treatment

Time	G1			S			G2/M		
	CT	RACT	LG153 CT	CT	RACT	LG153 CT	CT	RACT	LG153 CT
Day 1	21.2 ± 0.6	24.1 ± 0.2	20.9 ± 0.2	54.7 ± 0.8	49.9 ± 0.3	55.0 ± 2.0	24.1 ± 1.4	26.0 ± 0.1	24.1 ± 1.8
Day 2	21.4 ± 1.2	45.1 ± 0.6	24.9 ± 0.1	54.0 ± 1.2	42.3 ± 0.7	56.4 ± 0.4	24.6 ± 0.01	12.6 ± 0.2	18.8 ± 0.6
Day 3	26.6 ± 1.0	47.5 ± 0.5	38.9 ± 1.0	61.2 ± 6.4	39.3 ± 1.5	48.0 ± 0.2	12.2 ± 5.4	13.2 ± 0.9	13.1 ± 1.1
Day 4	27.8 ± 1.1	44.8 ± 0.1	43.4 ± 0.04	59.8 ± 1.9	38.8 ± 1.2	44.1 ± 0.6	12.4 ± 0.8	16.4 ± 1.3	12.5 ± 0.5

cle. A quantity of 4.5 ml prewarmed cell slurry mixture containing  $3.0 \times 10^6$  cells was poured on the preincubated bottom agar layer. Following a 6-h incubation (37°C, 10% CO<sub>2</sub>), 3.0 ml 1× DMEM supplemented as above was pipetted onto the second layer. Colonies were visible within 11 days.

**RNA isolation and blotting.** RNA isolation and northern blotting procedures are described in Li *et al.* [24].

**Preparation of protein extracts and Western blotting.** F9 cell monolayers were lysed on ice for 30 min in 50 mM Tris-HCl (pH 8), 120 mM NaCl, and 0.5% Triton X-100, containing a cocktail of protease and phosphatase inhibitors. Lysates were passed through a 26-gauge needle several times and then spun in a microcentrifuge for 15 min at 4°C to pellet cellular debris. The supernatants were frozen in liquid N<sub>2</sub> and stored at -80°C until used. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA).

For Western blotting, 20–25 µg protein was loaded on SDS-PAGE minigels [25]. Following electroblotting, filters were blocked in 5% nonfat dry milk and then exposed to antibodies overnight. Proteins were visualized by enhanced chemiluminescence (Amersham) and quantified by densitometry. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (cyclin D1, SC-450; cyclin D2, SC-593; cdk4, SC-260) and Neomarkers (Union City, CA) (p27, MS-256; cyclin D3, MS-215; and p21, MS-5387).

**Cyclin-dependent kinase assays.** Kinase assays were done essentially as described by DeGregori *et al.* [26]. Equivalent amounts of protein extract, as determined by Bradford assay (Bio-Rad), were then added to protein A/protein G agarose beads (Santa Cruz) that had been precoated with 2 µg polyclonal anti-Cdk4 (Santa Cruz SC-260) or polyclonal anti-Cdk6 (Santa Cruz SC-177) antibodies. Complexes were immunoprecipitated for 3 h at 4°C with constant rotation. Immunoprecipitates were washed and then resuspended in 30 µl kinase reaction mix (50 mM Hepes, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 µM ATP, and 10 µCi [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol, DuPont NEN, Boston, MA), containing 2 µg bacterially expressed GST-Rb (amino acids 379–928, kindly provided by Doug Cress, Muffitt Cancer Center) as substrate. After incubation at 30°C for 30 min, the reactions were stopped by the addition of SDS sample buffer and then boiled for 5 min, electrophoresed through 7.5% SDS-PAGE, and exposed to X-ray film. The amount of kinase activity was quantified by use of a phosphorimager (Molecular Dynamics).

## RESULTS

### All-trans-RACT Treatment Increases the Length of the F9 Cell Cycle

Cells induced to differentiate into parietal endoderm by exposure to all-trans-RA, 250 µM dbcAMP, and 500 µM theophylline (RACT) for 4 days grow much slower than undifferentiated cells. The decreased growth rate

and induction of complete differentiation depends on all-trans-RA, because cells treated only with 250 µM dbcAMP and 500 µM theophylline (CT) fail to differentiate and continue to divide rapidly. We compared the percentage of cells in each phase of the cell cycle during 4 days of treatment with CT or 0.1 µM all-trans-RACT by staining with propidium iodide and measuring DNA content by flow cytometry. Results are shown in Table 1. By the second day of treatment, 45% of the RACT-treated cells, compared to 21% of the CT-treated cells, were in G1 phase. All-trans-RACT treatment eventually caused the number of cells in S phase to decline from 60 to 39%. Thus, the decreased growth of all-trans-RACT-treated cells was largely due to an increase in the length of G1 phase relative to that of undifferentiated CT-treated cells.

### Regulation of Cyclin D1, D2, and D3 Transcripts in F9 Embryonal Carcinoma Cells

The cyclin D proteins are major regulatory subunits of the G1 Cdks. Since their concentrations directly regulate G1 progression, we measured the abundance of the cyclin D transcripts in F9 embryonal carcinoma cells induced to terminally differentiate into parietal endoderm *in vitro*. A time course of cyclin D expression was obtained by isolating RNA from cells treated with 0.1 µM all-trans-RA (Figs. 1A and 1B) or 1.0 µM all-trans-RA (Figs. 1C and 1D) and CT for 1, 2, 3, or 4 days. As controls, RNA was isolated from cells treated only with CT at the same time points. Figures 1A and 1C show the autoradiographs of Northern blots of this RNA and Figs. 1B and 1D show the data normalized to the constitutive message 36B4 [27].

Cyclin D1 mRNA abundance in cells induced to differentiate by exposure to 0.1 µM all-trans-RA and CT for 4 days was 26% of that observed in undifferentiated cells treated only with CT ( $P < 0.05$ ,  $n = 4$ , Figs. 1A and 1B, compare lanes 4 and 8; Fig. 4; and data not shown). A similar repression was observed in cells treated with 1.0 µM all-trans-RA and CT (Figs. 1C and 1D and data not shown). We also observed a decline in cyclin D1 mRNA levels during culture in CT-containing medium (Figs. 1A and B). Since all-trans-RACT-

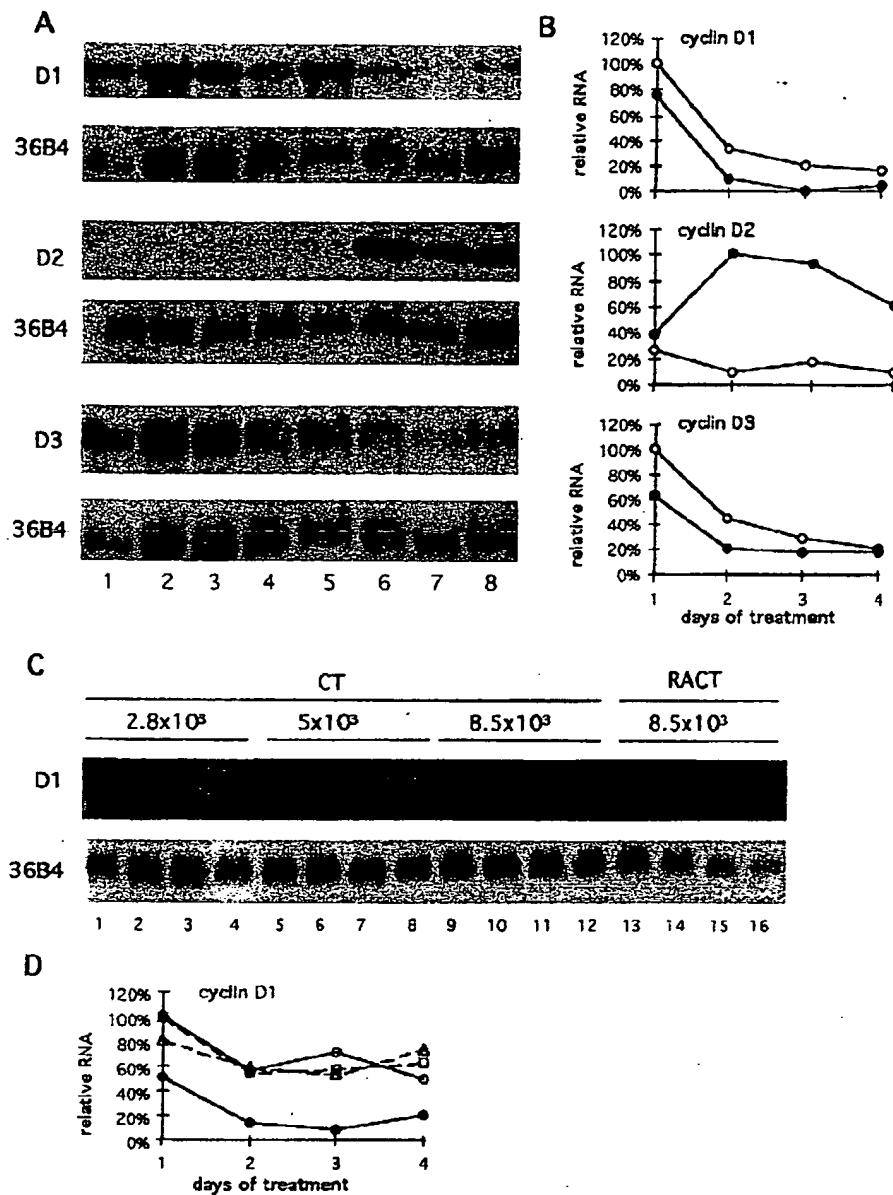


FIG. 1. Time course of cyclin D1-3 RNA levels during F9 cell differentiation. (A) Representative autoradiographs of Northern blots of RNA isolated from F9 cells seeded at  $8.5 \times 10^3$  cells/cm<sup>2</sup> and treated with 250  $\mu$ M dbcAMP and 500  $\mu$ M theophylline (CT, lanes 1-4) or CT with 0.1  $\mu$ M all-trans-RA (RACT, lanes 5-8) for 1 day (lanes 1, 5), 2 days (lanes 2, 6), 3 days (lanes 3, 7), or 4 days (lanes 4, 8). 25  $\mu$ g of RNA was loaded per lane. Blots were probed for the cyclin D1, D2, D3, and 36B4 RNAs as indicated. Similar results were obtained with three to four independent Northern analyses. (B) Graphs indicate the transcript levels shown in A normalized to the level of the constitutive RNA, 36B4. In each graph, RNA levels are expressed as a percentage of those observed in the cells having the highest abundance of that RNA. (C) RNA was isolated and analyzed for cyclin D1 RNA abundance exactly as in A and B except that cells were seeded in CT only (lanes 1-12) at  $2.8 \times 10^3$  cells/cm<sup>2</sup> (lanes 1-4),  $5 \times 10^3$  cells/cm<sup>2</sup> (lanes 5-8), or  $8.5 \times 10^3$  cells/cm<sup>2</sup> (lanes 9-12) or in 1  $\mu$ M all-trans-RACT seeded at  $8.5 \times 10^3$  cells/cm<sup>2</sup> (lanes 13-16). Cells were grown for 1 day (lanes 1, 5, 9, 13); 2 days (lanes 2, 6, 10, 14); 3 days (lanes 3, 7, 11, 15); or 4 days (lanes 4, 8, 12, 16). (D) Transcript levels shown in C normalized to the level of the constitutive RNA, 36B4. ●, RACT-treated, seeded at  $8.5 \times 10^3$  cells/cm<sup>2</sup>; □, CT-treated, seeded at  $8.5 \times 10^3$  cells/cm<sup>2</sup>; △, CT-treated, seeded at  $5 \times 10^3$  cells/cm<sup>2</sup>; ▲, CT-treated, seeded at  $2.8 \times 10^3$  cells/cm<sup>2</sup>.

treated cells have a decreased growth rate, CT-treated cultures seeded at the same density contain approximately three times as many cells after 4 days of cul-

ture. Therefore, we measured cyclin D1 levels in CT-treated cells seeded at the usual density of  $8.5 \times 10^3$  cells/cm<sup>2</sup> or at  $5 \times 10^3$  or  $2.8 \times 10^3$  cells/cm<sup>2</sup>. The data

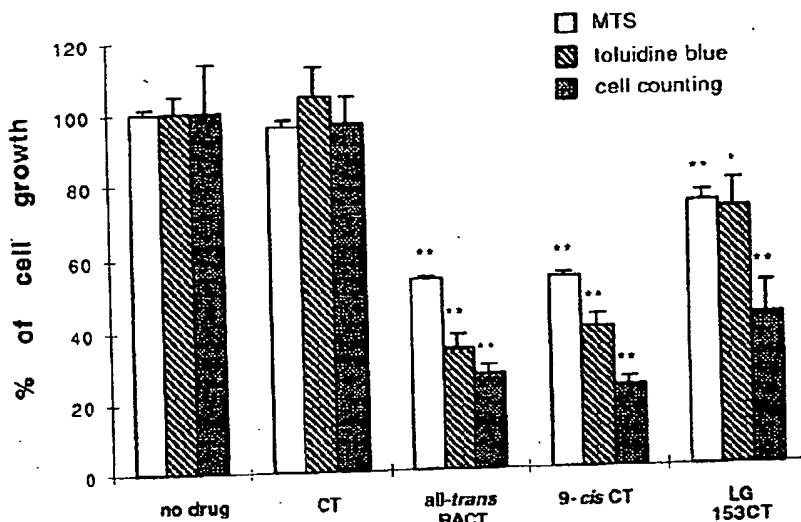


FIG. 2. The effects of various retinoids on F9 cell growth. Cells were grown 3 days in DMEM containing CT and 0.1  $\mu$ M retinoids as indicated under each bar. For the MTS assay, cells in 96-well plates were assayed by increased conversion to formazan and absorption at 570 nm was determined on microplate reader ( $n = 8$ ). For toluidine blue staining, absorption at 630 nm was measured in 96-well plates ( $n = 8$ ). The growth of cells in 24-well plates was measured by cell counting ( $n = 4$ ). The values were normalized relative to the number of untreated cells. Bars, SEM. \* $P < 0.05$ , \*\* $P < 0.01$  relative to undifferentiated (untreated or CT-treated) cells.

shown in Figs. 1C and 1D indicate that, although cyclin D1 levels may decline in cells grown at high density, all-trans-RACT further decreases the abundance of the cyclin D1 mRNA.

In contrast to the observed decrease in cyclin D1 mRNA level, 0.1  $\mu$ M all-trans-RA and CT induced the cyclin D2 RNA eightfold over that observed in CT-treated cells within 48 h of treatment ( $P = 0.005$ ,  $n = 4$ , Figs. 1A and 1B, lane 6; and data not shown). Likewise, 1.0  $\mu$ M all-trans-RA and CT induced the cyclin D2 RNA strongly by 48 h (data not shown). As observed for the cyclin D1 transcript, the abundance of the cyclin D3 RNA declined with culture in both all-trans-RACT and CT-containing medium (Fig. 1). Cell density failed to affect this decline (data not shown). Together, these data indicate that the cyclin D1 and D2 transcript levels are differentially regulated by all-trans-RACT during F9 cell growth and differentiation.

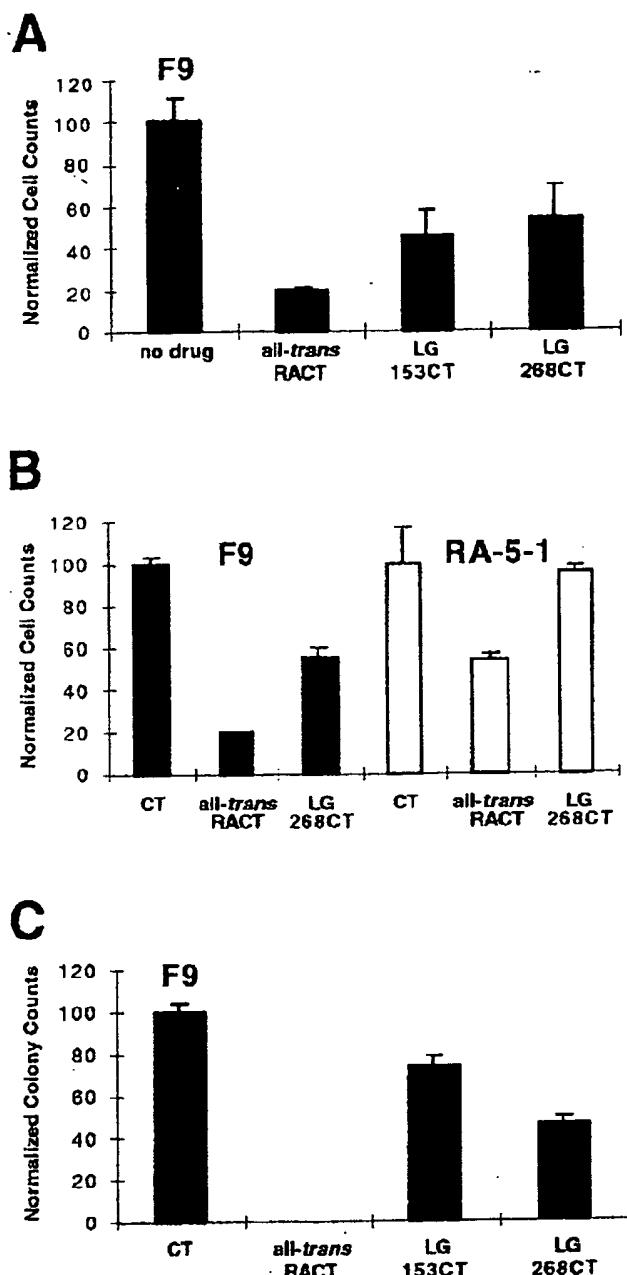
#### RXR Agonists Inhibit F9 Cell Proliferation

The decreased F9 cell growth rate caused by all-trans RA could involve genes regulated by factors associated with terminal differentiation. This could be an indirect retinoid effect, because many transcription factors are regulated by RA. Alternatively, all-trans-RA-bound receptors could directly regulate the cell cycle genes controlling growth rate. Most likely, a combination of these mechanisms plays a role. All-trans-RA, which directly activates RAR $\alpha$ ,  $\beta$ , and  $\gamma$ , may be metabolized to 9-cis-RA and thus indirectly activate the RXRs as well. One approach to distinguish direct

from indirect regulation of the cyclin D genes would require a laborious analysis of the genetic regulatory elements controlling each gene. An alternative pharmacological approach using receptor-selective retinoids is more suitable for analyzing many genes. These retinoids can activate a subset of receptors and thus a subset of the genes activated in all-trans-RA-treated F9 cells.

We compared the growth of F9 cells treated with 0.1  $\mu$ M all-trans-RA, an RAR agonist; 9-cis-RA, an RAR and RXR panagonist; and LG100153 (LG153), an RXR agonist [18]. Three independent cell proliferation assays were used to measure cell growth (Fig. 2). Each assay showed that CT treatment alone did not change the growth rate relative to untreated F9 cells. In contrast, exposure to 0.1  $\mu$ M all-trans-RA or 9-cis RA and CT resulted in significantly fewer cells (Figs. 2 and 3A). The RXR agonist LG153 and CT also repressed the number of cells by 50%, although the morphology typical of differentiated cells was not induced. The difference in growth was statistically significant as determined by the Student's  $t$  test assuming equal variances (MTS assay,  $P < 0.01$ ; cell counting,  $P < 0.01$ ; toluidine blue assay,  $P < 0.05$ ). Since LG153 specifically activates RXRs, these observations suggest that ligand activation of RXRs by LG153 inhibits the growth of F9 cells. LG153 has also been observed to block the cell cycle of the myelomonocytic cell line U937 [28].

To examine the effect of LG153 activation on cell cycle traverse, we determined the percentage of cells in each phase of the cell cycle by flow cytometry. As shown



**FIG. 3.** RXR activation selectively inhibits F9 but not RA-5-1 cell growth. F9 (A, B) or RA-5-1 (B) cells were seeded at  $2 \times 10^4$  cells per well in 24-well plates and grown for 4 days in medium containing 0.1  $\mu$ M retinoid and CT as indicated. Cells were then trypsinized and counted on a hemacytometer ( $n = 3-4$ ). (C) F9 cells were grown in semisolid agarose in medium containing 0.1  $\mu$ M retinoid and CT as indicated. After 11 days, visible colonies were counted ( $n = 9-13$ ). All values are normalized to the number of cells or colonies in the CT-treated cultures. There was no difference in the size of colonies formed in CT or RXR-selective retinoids. Solid bars, F9 cells; open bars, RA-5-1 cells. Bars, SEM.

in Table 1, treatment with LG153 and CT for 4 days caused an increase in the number of cells in G1 phase from 21 to 43%. This did not differ significantly from the 45% G1 phase cells found in the RACT-treated cultures. The kinetics of this change in G1 length were different. RACT nearly doubled the number of G1 cells within 48 h of treatment. In contrast, the number of LG153-treated cells in G1 phase were not significantly different until 72 h of treatment. Thus, while RXR activation can inhibit the growth of F9 cells, the kinetics of this inhibition are distinct from that induced by RAR activation.

To determine whether or not the significant inhibition of F9 cell growth rate by LG153 was caused by the selective activation of RXRs, we tested another RXR-selective agonist, LG100268 (LG268) [19]. Figure 3A demonstrates that 4 days of exposure to 0.1  $\mu$ M LG153 or LG268 and CT repressed F9 cell growth by 49 and 47% respectively. The average doubling times of the cells over 4 days were 16.6 h in the absence of retinoids, 27.8 h in all-trans-RACT, 19.8 h in LG153CT, and 20.7 h in LG268CT-containing medium. The receptor selectivity of these retinoids has been confirmed by binding studies and mammalian reporter assays [18, 19]. Therefore, our observations are consistent with the growth inhibition resulting from the ability of each drug to activate RXRs.

*The RXR-Selective Agonist LG268 Fails to Inhibit the Proliferation of an F9 Cell Line That Cannot Differentiate*

Wang and Gudas isolated a mutant clone of F9 cells, RA-5-1, that cannot differentiate into either parietal [20] or visceral endoderm [29]. These cells, which have a defective prolyl-4-hydroxylase enzyme, were selected by virtue of their ability to grow without anchorage in medium containing all-trans-RA [30]. All-trans-RA fails to induce differentiation or complete growth arrest in RA-5-1 cells, although *Hoxa-1*, a directly RA-responsive gene, is induced [29]. Thus RA-5-1 cells begin, but fail to complete, the series of events leading to terminal differentiation. To test whether or not the RXR-selective agonists were nonselectively cytotoxic, we examined the effect of LG268 on RA-5-1 growth. As shown in Fig. 3B, all-trans-RACT causes a 47% decrease in RA-5-1 growth rate. This is significantly less than the 80% decrease observed in wild-type F9 cells (Fig. 3B, compare bars 2 and 5) and is consistent with published data [30]. In contrast, LG268CT did not alter the growth of RA-5-1 cells (Fig. 3B, compare bars 4 and 6), although this drug inhibited F9 cell growth by 45%. This proves that LG268 is not generally cytotoxic. This result also suggests that RXR-selective retinoids inhibit cell growth by a mechanism that is impaired in RA-5-1 cells.

*RXR-Selective Retinoids Inhibit, but Do Not Abolish, Anchorage-Independent Growth*

The previous results indicate that LG153 and LG268 decrease F9 cell growth rate in monolayer cultures. F9 cells induced to differentiate by all-trans-RACT are, like most benign cells, incapable of anchorage-independent growth. In contrast, untreated F9 embryonal carcinoma cells are highly malignant and readily form colonies in semisolid agar. To further assess the effects of the RXR-selective retinoids on growth control in F9 cells, we compared the number of colonies formed in soft agar containing 0.1  $\mu$ M LG153, LG268, or all-trans-RA and CT. Cells ( $3 \times 10^3$ ) were plated in 0.35% agar and colonies were counted after 11 days. Cells treated with CT alone formed an average of  $520 \pm 78$  colonies per plate. As expected for terminally differentiated cells, all-trans-RACT-treated cells failed to form colonies in soft agar. LG153CT- and LG268CT-treated cells formed  $385 \pm 82$  and  $242 \pm 51$  colonies per plate, respectively. Figure 3C shows the number of colonies normalized to the average number of colonies in CT-treated plates. Thus, both RXR-selective retinoids impair colony formation, although not to the same extent as all-trans-RA. These data are consistent with two hypotheses: (1) a subset of RXR agonist-treated cells terminally differentiates and these fail to form colonies, or (2) the RXR agonists fail to induce complete terminal differentiation, but cause some cells to become anchorage-dependent through partial differentiation or another mechanism. We do not favor the first hypothesis, because the RXR agonist-treated cells did not assume the characteristic parietal endoderm morphology. Also, as described below, the pattern of gene expression in RXR agonist-treated cells resembles that of undifferentiated cells.

*LG153-Treated Cells Express Undifferentiated Stem Cell Markers*

All-trans-RA regulates cell differentiation and proliferation by modulating gene expression [31]. To learn which genes are involved in RXR-mediated growth inhibition, we used Northern analyses to quantify the expression of several genes known to be regulated during all-trans-RA-induced F9 cell differentiation (Fig. 4). These genes included those expressed in undifferentiated stem cells and down-regulated by all-trans-RA, as well as those induced by all-trans-RA. Previous work showed that LG153 does not affect the expression of *H218*, which functions as a receptor for sphingosine 1-phosphate and related compounds and is down-regulated during RA-induced differentiation [24]. We also tested five other genes normally expressed in undifferentiated cells: the transcription factors *Rex1* (*Zfp-42*) [32], *Oct3/4* [33], and *Sox2* [34] and the growth factors *Bmp4* [13] and *Fgf4* [35]. As expected, each gene

was down-regulated by RAR agonists (all-trans-RA, TTNPB, or 9-cis RA) but not by the RXR-selective agonist LG153. TTNPB activates RARs highly selectively and cannot be metabolized to 9-cis-RA.

LG153 also failed to fully up-regulate the expression of the genes encoding the extracellular matrix protein *laminin B1*, the growth factor *Bmp2*, or the transcription factors *Hoxa-1* and *RAR $\beta$* , as occurs in differentiated cells [32 and Refs. therein]. If the 25 to 50% decreases in colony formation shown in Fig. 3C were caused by terminal differentiation of 25 to 50% of the cells, then a corresponding decrease in the expression of all stem cell markers and an increase in all parietal endoderm markers should have been observed. Since this was not observed, LG153 does not appear to induce parietal endoderm in a subset of the F9 cell population.

*LG153 Induces Cyclin D2 and D3 mRNA*

All-trans-RA slows the F9 cell cycle by increasing the length of the G1 phase (Table 1) [17, 36]. We also observed that cyclins D1, D2, and D3 were regulated during F9 cell differentiation (Fig. 1). Therefore, we measured the expression of RNAs encoding G1 phase regulatory proteins which are directly involved in the cell cycle and growth control in cells treated with a panel of retinoids (Fig. 5). Retinoids that induced differentiation (all-trans-RA, TTNPB, and 9-cis-RA) down-regulated *cyclin D1* and *cyclin E*. Conversely, as observed for the stem cell markers *H218*, *Rex1*, *Oct3/4*, *Sox2*, *Bmp4*, and *Fgf4*, the levels of *cyclin D1* and *E* RNAs were unaltered in LG153-treated cells. Thus regulation of these genes is linked to RAR-activation and parietal endoderm differentiation.

Cyclin D2 and D3 message abundance, however, was changed in RXR agonist-treated cells. Both LG153 (Fig. 5) and LG268 (data not shown) induced cyclin D2 RNA levels as efficiently as RAR agonists or panagonist (8-fold). Further, cyclin D3 mRNA levels were induced by the RXR agonist LG153, but not by all-trans-RACT. The RNA-encoding p27<sup>Kip</sup>, a major Cdk inhibitor, was modestly, but reproducibly, up-regulated by both all-trans-RACT (2-fold,  $P < 0.01$ ,  $n = 6$ ) and LG153CT (1.4-fold,  $P < 0.05$ ,  $n = 6$ ). These observations suggest that cyclin D2, cyclin D3, and p27<sup>Kip</sup> may be involved in the partial growth suppression caused by RXR agonists. More importantly, the striking elevation of cyclin D2 levels during all-trans-RACT-induced differentiation may not be involved in differentiation, but rather in cell cycle exit.

*RAR- and RXR-Selective Retinoids Differentially Regulate Cell Cycle Protein Levels*

Having shown distinct modulations of mRNAs encoding cell cycle proteins upon treatment with RAR or RXR-selective retinoids, we next examined the relative

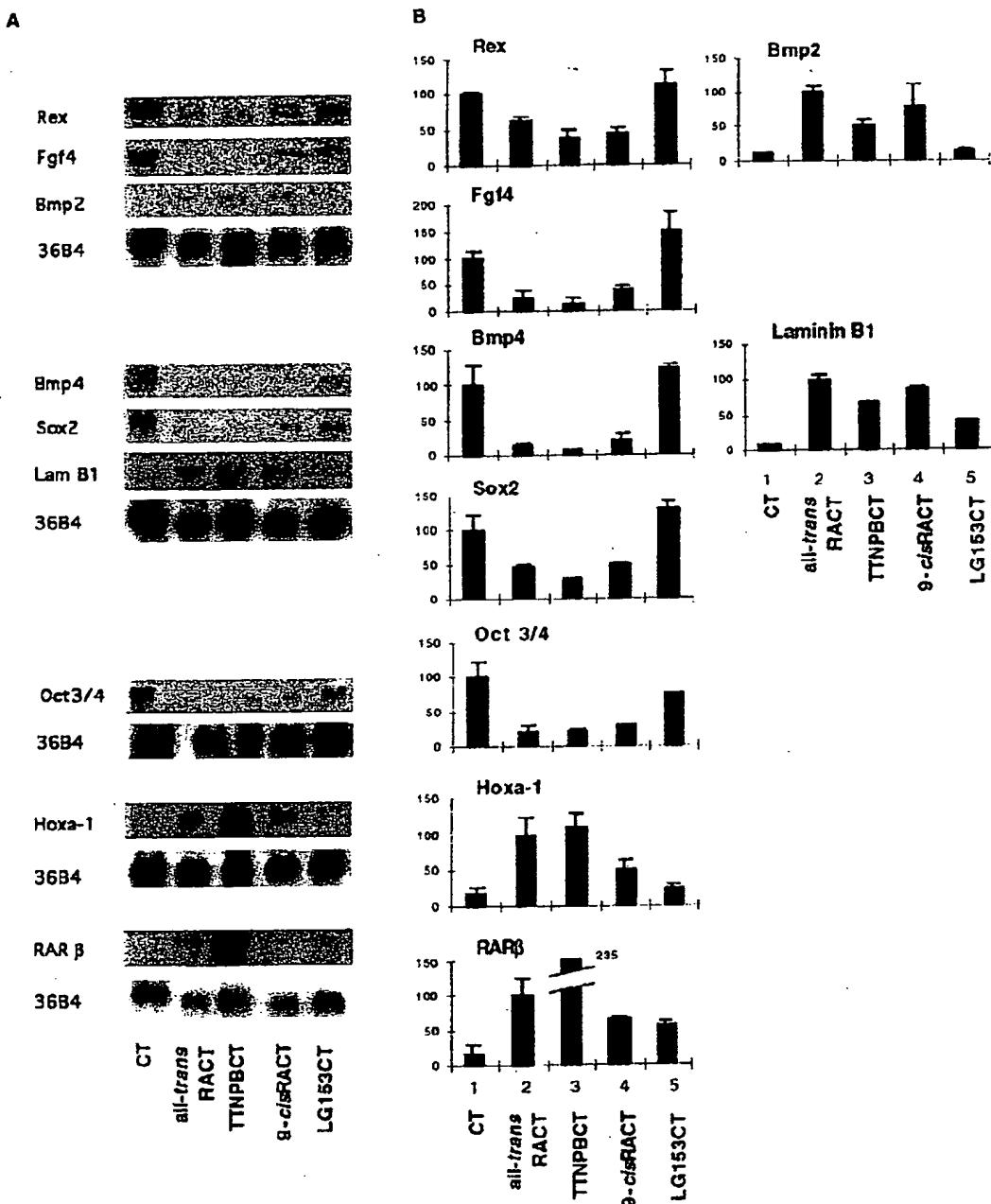


FIG. 4. Northern analyses of genes expressed in F9 cells treated with different retinoids. (A) Representative autoradiographs of Northern blots of RNA isolated from F9 cells treated for 102 h with CT and 0.1  $\mu$ M retinoids as indicated at the bottom of each lane. 22  $\mu$ g total cellular RNA per lane was loaded on formaldehyde-containing agarose gels. Following electrophoresis, RNA was transferred to nylon filters and hybridized to the indicated radiolabeled cDNA probes (Lam B1, *Laminin B1*). The autoradiographs of the representative blots hybridized to the constitutive RNA, 36B4, is shown at the bottom of each set of blots. (B) The amount of signal was quantified on a phosphorimager. Bar graphs indicate a compilation of the data from several Northern blots, normalized to 36B4. Loading variation between lanes was less than threefold.  $n = 2$  for all blots, except  $n = 4$  for *Hoxa-1*. The mRNA levels are expressed as a percentage of the average value observed in cells treated with CT alone (down-regulated genes: *Rex1*, *Oct 3/4*, *Fgf4*, *Bmp4*, and *Sox2*) or all-trans-RACT (up-regulated genes: *Bmp2*, *Hoxa-1*, *laminin B1*, and *RAR $\beta$* ). Bars, range or SEM.

protein levels directly (Fig. 6A). The amount of cyclin D1 protein in all-trans-RACT-treated cells was approximately one-half the amount in undifferentiated cells

(Fig. 6A). In contrast, treatment with the RXR-selective retinoid (LG268CT) did not cause any appreciable change in the amount of cyclin D1 protein. While the

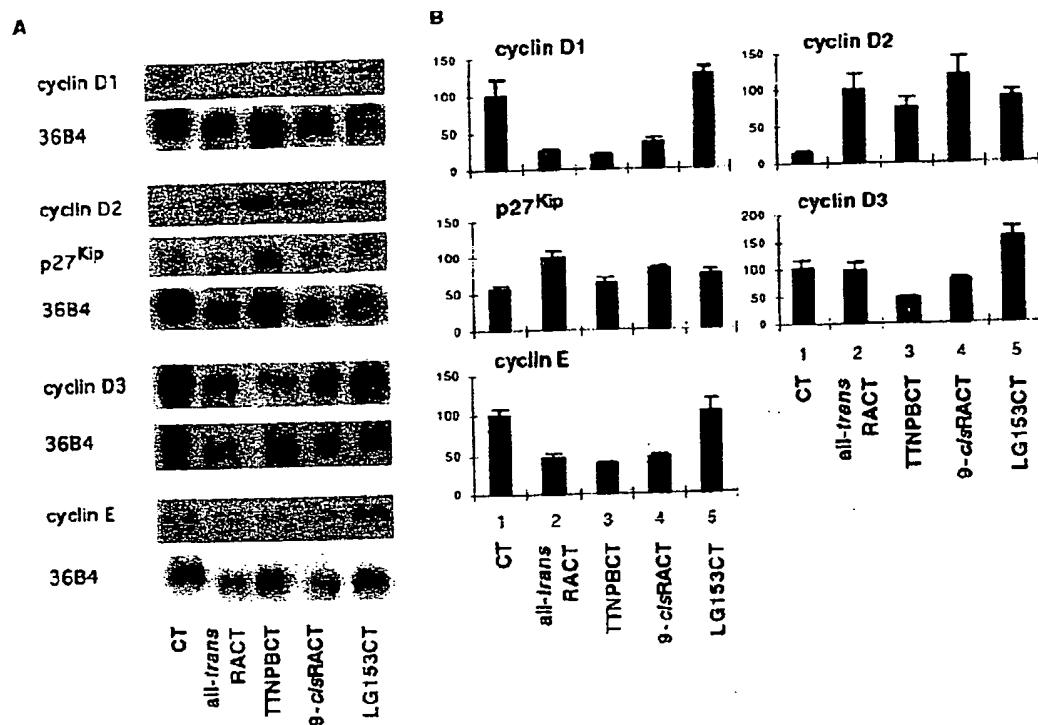


FIG. 5. Cell cycle mRNA levels in F9 cells treated with different retinoids. (A) Representative autoradiographs of Northern blots of RNA isolated from F9 cells treated with CT and retinoids as indicated for Fig. 4. (B) Bar graphs indicate a compilation of the data from several Northern blots, normalized to 36B4.  $n = 6$  for  $p27^{Kip}$ , cyclin D1, and D2.  $n = 2$  for cyclin D3 and cyclin E.

cyclin D2 protein was barely detectable in undifferentiated cells, cyclin D2 protein levels were strongly induced by all-trans-RACT. Treatment with LG268CT induced cyclin D2 expression to a level almost one-half that of all-trans RACT. Cyclin D3 levels declined by approximately 40% upon treatment with all-trans-RACT, but were induced threefold by the RXR-selective retinoid and CT. Thus, overall cyclin D levels increased in RXR-treated cells, despite their decreased rate of proliferation.

Cdk4 protein levels did not change appreciably following any treatment (Fig. 6A). In contrast to treatment with all-trans-RACT which induced  $p27^{Kip}$ , RXR-selective agonist and CT did not change the abundance of the Cdk inhibitors p21 or  $p27^{Kip}$  (Fig. 6B). Thus, in RXR agonist-treated cells, the levels of cyclin D1, p21, and  $p27^{Kip}$  proteins resemble those of undifferentiated cells, while cyclin D2 and D3 protein levels are specifically induced.

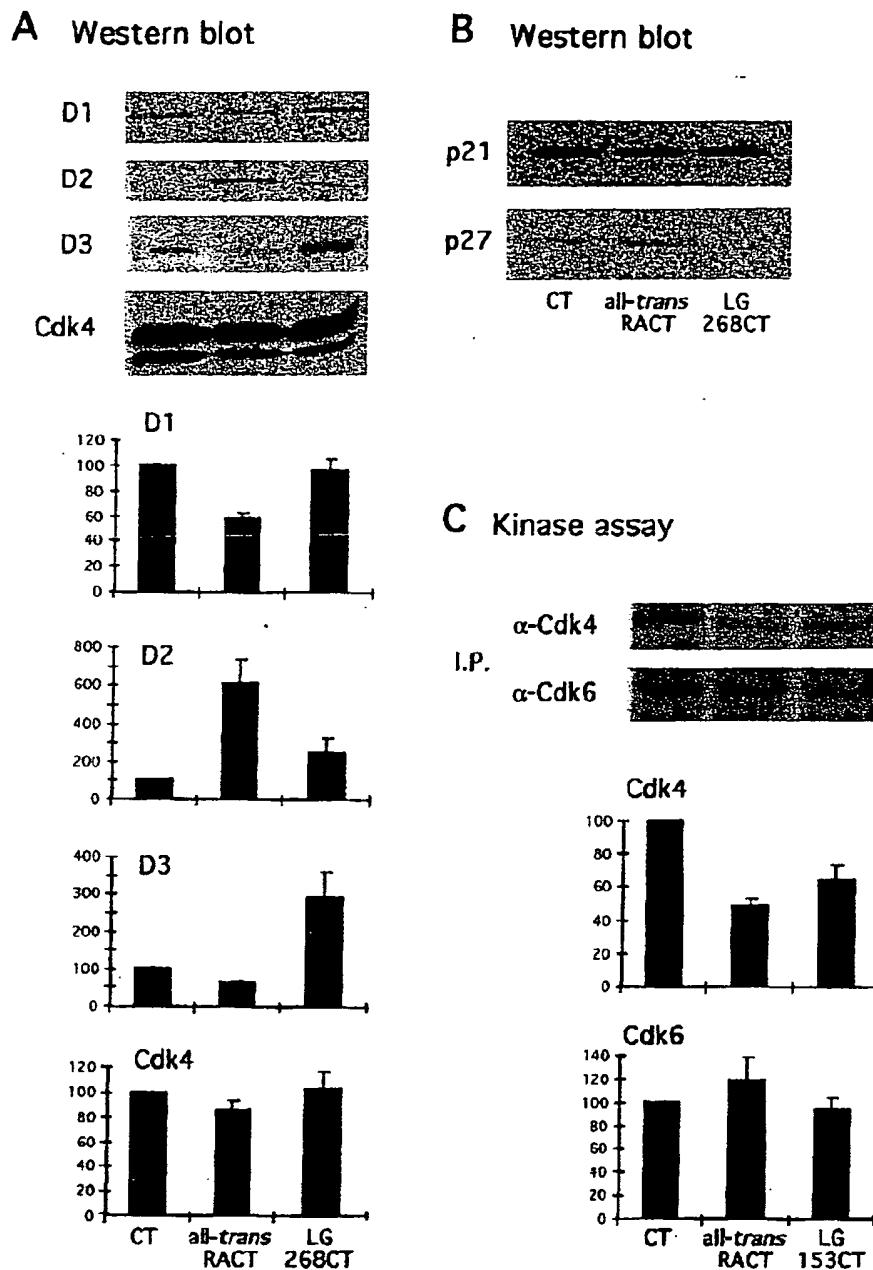
#### Like All-trans-RA, Treatment with RXR-Selective Retinoid Decreases Cdk4-Dependent Kinase Activity

Having shown that RXR-selective retinoids modulate the expression of D-type cyclins in a manner distinct from that of all-trans-RA, we next examined the functional consequences of this regulation. In extracts prepared from all-trans-RACT-treated cells, the

amount of Cdk4-dependent kinase activity was 50% that observed in cells treated with CT alone (Fig. 6C). In these fully differentiated cells, decreased Cdk4 kinase activity correlates with decreased proliferation rate. RXR-selective retinoids, which cannot cause full differentiation (Fig. 4), also impair proliferation (Figs. 2 and 3). *In vitro* kinase assays showed that treatment with the RXR-selective retinoid LG153 and CT decreased Cdk4-dependent kinase activity by 36% (Fig. 6C). Cdk6-dependent kinase activity was not appreciably affected by any treatment. The magnitude of the LG153-associated decline in Cdk4 activity correlates with the antiproliferative effect of this retinoid.

#### DISCUSSION

The retinoid-induced complete differentiation of F9 embryonal carcinoma stem cells into parietal endoderm is accompanied by a sharply decreased proliferation rate as the cells become benign (Table 1) [17, 36]. Changes occur in numerous cellular systems during this process. Cells activate and inactivate various transcription factor genes, change shape and adhesive properties, and begin to secrete specialized extracellular matrix proteins. Some of these changes result from the direct regulation of specific genes by retinoids and



**FIG. 6.** Cell cycle protein levels and Cdk activity in retinoid-treated cells. F9 cells were treated with CT alone or 1  $\mu$ M of the indicated retinoid and CT for 3 or 4 days. Representative autoradiographs of ECL-visualized Western blots (A, B) or phosphorylated GST-Rb (C) are shown. (A and B) 25  $\mu$ g of whole cell extract was electrophoresed through 12% (A) or 15% (B) SDS-PAGE gels and then the proteins were electroblotted to nitrocellulose. Blots were incubated with antibodies to the indicated proteins and were visualized by enhanced chemiluminescence. Protein levels were quantitated by densitometry and expressed as a percentage of the value observed in cells treated with CT alone. Data represent two to four experiments; bars, range or SEM. (C) Whole cell extracts were immunoprecipitated with antibodies to Cdk4 or Cdk6. Immunoprecipitates were then tested for their ability to phosphorylate bacterially produced GST-Rb. Reactions were electrophoresed on 7.5% SDS-PAGE gels. Kinase activity was quantitated with a phosphorimager and expressed relative to the kinase activity measured in CT-treated cells. Extracts immunoprecipitated with normal rabbit serum were not capable of phosphorylating Rb, indicating the specificity of the assay (data not shown). Data represent four experiments. Bars, SEM. RACT- and LGCT-treated cells differed significantly from the CT-treated cells in the cdk4 kinase assay ( $P < 0.01$ ). For the Cdk6 kinase assay, none of the samples differed significantly.

their receptors, e.g., *Hoxa-1* [37, 38], while others are secondarily regulated by the products of directly regulated genes encoding transcription factors. The decreased growth rate of RA-treated F9 cells could result from direct regulation of cell cycle genes or by other genes regulated during parietal endoderm differentiation. We have presented an analysis of the changing expression levels of key cell cycle regulatory genes, cyclins D1, D2, D3, and E, and p27<sup>kip</sup>, during F9 cell differentiation. We also present evidence that a retinoid-stimulated decrease in proliferation can be unlinked from terminal differentiation.

We observed that two different RXR agonists increased the doubling time of F9 cells from 16.6 to 20 h (Figs. 2 and 3). RXR activation can also slow proliferation, without inducing differentiation of a melanoma cell line [39]. The inhibition of Cdk4 activity we observed in F9 cells (Fig. 6C) might explain this decreased growth rate. Unexpectedly, however, cyclin D2 and D3 protein levels were induced in F9 cells treated with RXR agonist (Fig. 6A). The best characterized function of the cyclin Ds is to activate Cdk4 and Cdk6. However, recent observations support a distinct role for the G1 cyclins in differentiated cells [40]. Cyclin D2 or D3 have been shown to enter into unique protein complexes in cells exiting the cell cycle. For example, cyclin D3 bound both cyclin E and p130 in differentiated myotubes [41] and cyclin D2 bound catalytically inactive Cdk2 in fibroblasts entering quiescence [42]. In fact, overexpression of cyclin D2 alone inhibited G1 progression. An increase in cyclin D3 protein abundance has also been recently reported as a relatively early event in HL-60 cell differentiation [43]. Whether or not the induction of cyclin D2 and D3 is required for the RXR-mediated growth suppression remains to be tested. However, the fact that RXR-selective retinoids can inhibit proliferation and induce cyclin D2 without inducing the complex differentiation program suggests that the induction of cyclin D2 during all-trans-RACT-induced differentiation may be involved in cell cycle exit rather than differentiation *per se*.

Retinoids inhibit the proliferation of most cells; however, some normal and malignant cells are stimulated to proliferate by retinoids or require retinoids for survival [see 44 and Refs. therein]. Indeed, supplements of  $\beta$ -carotene, a major dietary precursor to vitamin A, were found to increase the cancer rate in smokers [45], suggesting that some retinoids promote tumor cell survival *in vivo*. Undifferentiated F9 cells may possess a repressive function that prevents growth despite retinoid-induced upregulation of cyclin D2 and D3. This function may be absent in cells that require retinoids for survival.

The complete differentiation of parietal or visceral endoderm from F9 embryonal carcinoma stem cells is a multistep process initiated by various retinoids that

activate RARs [13]. Retinoids that activate the RXRs do not induce differentiation alone, but can synergize with suboptimal RAR agonist concentrations to cause complete differentiation [46, 47]. During F9 cell differentiation, the expression of numerous genes is up-regulated or down-regulated [31]. We tested the anti-proliferative effects of an RXR agonist on an F9 cell line that is RA responsive, but cannot differentiate. RA-5-1 cells transcribe *Hoxa-1* in response to all-trans-RA, but fail to terminally differentiate or completely growth arrest [20, 29]. All-trans-RA-treated RA-5-1 cells grew slower than untreated cells, but the response was limited relative to that of wild-type F9 cells (Fig. 3B). In contrast, the RXR agonist did not alter RA-5-1 cell growth. Since RA-5-1 cells were selected by their mutant phenotype of growth in soft agar containing all-trans-RA [20] and since they initiate, but fail to complete, the differentiation program [20, 29], RXR-mediated decreased growth rate may require a process that is impaired in these cells.

Normal F9 cell differentiation clearly requires the activity of both RARs and RXRs, because F9 cells lacking functional RARs or RXRs were impaired in their ability to differentiate in response to all-trans-RA [17, 48, 49]. Interestingly, the phenotypes of these cell lines suggested that regulation of proliferation by retinoids was partially separable from differentiation. Although all-trans-RA and other retinoids failed to induce the normal gene markers of differentiation in RAR $\gamma$ -null cells, these cells were as sensitive as wild-type cells to the antiproliferative effects of the retinoids [17, 48]. In contrast, the RXR $\alpha$ -null cells also failed to differentiate, but resisted the antiproliferative effects of the retinoids [17]. Likewise, dominant-negative RXR mutant proteins inhibited RA-induced growth arrest in F9 cells [50] and RXR $\alpha$ -null cells proliferated more rapidly in the absence of retinoid [17]. Our observation that two chemically distinct RXR-selective ligands decrease the rate of F9 cell proliferation is consistent with the hypothesis that an RXR-mediated event directly influences cell cycle control in F9 cells. Additionally, the antiproliferative effect of retinoids does not require activation of the entire differentiation program.

The majority of retinoid-regulated genes are efficiently activated by ligand-bound RAR in a heterodimer with an unbound RXR [4, 7]. At least one dozen, however, bind and are activated by RXR homodimers *in vivo* and *in vitro* [7, 51, 52]. The RXRs also bind other hydrophobic ligand receptors such as the thyroid hormone, vitamin D, oxysterol, and peroxisome proliferator-activated receptors and Nurr77/NGFI-B [8–10]. One of the RXR-agonists with antiproliferative effects on our F9 cells (LG268) has been shown to directly stimulate the expression of 25-hydroxy-vitamin D3-24-hydroxylase, a key enzyme catalyzing 1,25-dihydroxyvitamin D metabolism, in reporter assays and in mice [52]. RAR $\beta$  has been previously shown to

be induced by RXR homodimers *in vitro* [6] and in yeast [53] and by RXR agonist in chick limb buds [54] and all-*trans*-RA-resistant breast cancer cells [55]. We also observed that the RXR agonist induced RAR $\beta$  modestly in F9 cells (Fig. 4). Finally, we have shown here that RXR agonists induced the abundance of the cyclin D2 and D3 RNAs and proteins without RAR agonist (Figs. 5 and 6), suggesting that RXRs may activate these important cell cycle genes by a mechanism independent of ligand-bound RARs.

The evidence indicates that all-*trans*-RA exerts its effects mainly via RAR/RXR heterodimers [4]. However, the ability of RXR ligands to alter the level of key proteins in the metabolic and signaling pathways of diverse signals, such as growth hormone, retinoids, and vitamin D [51–53], suggests that they can unexpectedly alter the response of cells to other ligands and thus influence proliferation. Our work also indicates that RXR ligands can directly influence the cell cycle apparatus.

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# The Geranylgeranyltransferase I Inhibitor GGTI-298 Induces Hypophosphorylation of Retinoblastoma and Partner Switching of Cyclin-dependent Kinase Inhibitors

A POTENTIAL MECHANISM FOR GGTI-298 ANTITUMOR ACTIVITY\*

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The geranylgeranyltransferase I inhibitor GGTI-298 has recently been shown to arrest human tumor cells in the G<sub>1</sub> phase of the cell cycle, induce apoptosis, and inhibit tumor growth in nude mice. In the present manuscript, we provide a possible mechanism by which GGTI-298 mediates its tumor growth arrest. Treatment of the human lung carcinoma cell line Calu-1 with GGTI-298 results in inhibition of the phosphorylation of retinoblastoma protein, a critical step for G<sub>1</sub>/S transition. The kinase activities of two G<sub>1</sub>/S cyclin-dependent kinases, CDK2 and CDK4, are inhibited in Calu-1 cells treated with GGTI-298. Furthermore, GGTI-298 has little effect on the expression levels of CDK2, CDK4, CDK6, cyclins D1 and E, but decreases the levels of cyclin A. GGTI-298 increases the levels of the cyclin-dependent kinase inhibitors p21 and p15 and had little effect on those of p27 and p16. Most interesting is the ability of GGTI-298 to induce partner switching for several CDK inhibitors. GGTI-298 promotes binding of p21 and p27 to CDK2 while decreasing their binding to CDK6. Reversal of partner switching and G<sub>1</sub> block was observed after removal of GGTI-298. Furthermore, GGTI-298 treatment results in an increased binding of p15 to CDK4, which is paralleled with decreased binding to p27. The results demonstrate that the GGTI-298-mediated G<sub>1</sub> block in Calu-1 cells involves increased expression and partner switching of CDK inhibitors resulting in inhibition of CDK2 and CDK4, and retinoblastoma protein phosphorylation.

in CAAX (C = Cys, A = aliphatic amino acid, X = any amino acid) at their carboxyl-terminal with GGTase I preferring leucine or isoleucine and FTase preferring Met or Ser in the X position (1). GGTase II, on the other hand, geranylgeranylates on two cysteines in proteins that end in XXCC, CCXX, or CXC sequences. Many prenylated proteins are small G-proteins that are integral components of proliferative signal transduction pathways (1). For example, Ras farnesylation is required for its ability to transduce growth signals from receptor tyrosine kinases to transcription factors and the cell cycle machinery that regulates cell division (2). Furthermore, mutated Ras is found in about 30% of all human cancers and is believed to cause malignant transformation by constitutive activation of abnormal growth (3). Farnesylation of oncogenic Ras is required for its cancer-causing activity (4). Similarly, the Rho family of small G-proteins such as RhoA and Rac1 require geranylgeranylation for their biological function. One of their key biological roles is to allow cells to traverse the G<sub>1</sub> phase of the cell cycle and begin DNA synthesis in S phase (5). Recently, RhoA and Rac1 have been shown to be required for malignant transformation by Ras (6, 7). Furthermore, constitutively activated RhoA and Rac1 can also lead to oncogenic transformation (6, 7). The overwhelming evidence implicating the Ras and Rho family of proteins in aberrant proliferative pathological conditions such as cancer and cardiovascular diseases prompted us and others (reviewed in Ref. 8) to design and synthesize FTase and GGTase I inhibitors.

FTI-277 and GGTI-298 are CAAX peptidomimetics that potently and selectively inhibit FTase and GGTase I, respectively (9, 10). We have found FTI-276 and its methyl ester FTI-277 to be potent inhibitors of oncogenic Ras processing and signaling (9). FTI-276 also potently inhibited the growth in nude mice of human tumors with multiple genetic alterations such as K-Ras mutation and p53 deletion (11, 12). FTIs from several other groups have also shown potent antitumor efficacy without toxicity in several animal models (8). Furthermore, several FTIs are presently in phase I clinical trials (13).

Selective inhibition of protein geranylgeranylation with GGTI-298 has major consequences on several biological pathways. Pretreatment of fibroblasts with GGTI-298, blocks PDGF- and epidermal growth factor-dependent tyrosine phosphorylation of their corresponding tyrosine kinase receptors (14). In contrast, selective inhibition of protein farnesylation has no effect on receptor tyrosine kinase phosphorylation (14). Furthermore, GGTI-298 inhibits the growth in nude mice of

Protein prenylation is an important posttranslational modification that is required for cellular localization and biological function of many proteins (1). These covalent attachments of farnesyl (C<sub>15</sub>) or geranylgeranyl (C<sub>20</sub>) to cysteines at the carboxyl-terminal of some proteins are catalyzed by three different enzymes. Farnesyltransferase (FTase)<sup>1</sup> and geranylgeranyltransferase I (GGTase I) modify cysteines of proteins that end

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<sup>1</sup> The abbreviations used are: FTase, farnesyltransferase; GGTase I, geranylgeranyltransferase I; GGTI, geranylgeranyltransferase inhibitor; FTI, farnesyltransferase inhibitor; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; CKI, cyclin-dependent kinase inhibitor;

PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis.

human tumors by a mechanism that is not yet known (12). One possible mechanism may involve GGTI-298-mediated G<sub>1</sub> phase block and apoptosis in cultured human tumor cells (15). The ability of GGTI-298 to inhibit proliferation is not limited to human tumor cells that are of epithelial and fibroblast origin. GGTI-298 also has a major effect on the proliferative pathways of smooth muscle cells (16). For example, GGTI-298 is very effective at inducing G<sub>1</sub> arrest and apoptosis in rat pulmonary artery smooth muscle cells. Furthermore, GGTI-298 enhances the ability of interleukin-1 $\beta$  to induce nitric oxide synthase-2 in the same cells (17). This induction of nitric oxide synthase-2 results in a large increase in the production of the nitric oxide radical, which is known to be inhibitory to smooth muscle cell proliferation. GGTI-298 also inhibited the ability of PDGF, interleukin-1 $\beta$ , and activated Ras to induce superoxide production in smooth muscle cells (18). Taken together, the data indicate that GGTI-298 has antiproliferative effects on fibroblasts, epithelial, and smooth muscle cells, and this cell growth inhibition appears to be mediated through a G<sub>1</sub> phase arrest. Recently, we have shown that in human tumors this G<sub>1</sub> arrest correlated with an induction of a cyclin-dependent kinase inhibitor p21<sup>WAF1</sup> (19). However, whether this induction of p21<sup>WAF1</sup> is responsible for the G<sub>1</sub> arrest is not yet known.

The G<sub>1</sub> to S phase transition of the mammalian cell division cycle is a highly regulated step. The key regulators of G<sub>1</sub>/S progression are a series of kinases that depend on cyclins for activation (for review, see Refs. 20–22). For example, the D-type cyclins are made in early G<sub>1</sub>, bind to and activate the G<sub>1</sub> cyclin-dependent kinases CDK4 and CDK6. Activation of these kinases also requires phosphorylation by CDK kinases such as CAK or cyclin H-CDK7 and dephosphorylation by phosphatases such as cdc25 (20). At this stage, the cell has reached a critical checkpoint of its cell cycle called the restriction point (R) at which time the cell checks that all is ready for DNA synthesis. Cyclin D-CDK4 and cyclin D-CDK6 complexes are now active and phosphorylate the retinoblastoma gene product pRb. Phosphorylated pRb disassociates from the transcription factor E2F, which, once freed from pRb, is able to induce the expression of several genes that prepare the cell for DNA synthesis (20). Among these is cyclin E, which activates CDK2, a kinase required for late G<sub>1</sub> to early S phase transition. The cyclin E-CDK2 complex hyperphosphorylates pRb, and the cell proceeds into S phase. At this time cyclin A expression is high, whereas cyclins D and E have been degraded (20). The cyclin A-CDK2 complex maintains the phosphorylation of pRb to sustain DNA replication. Finally CDK kinase activities are highly regulated by two families of CDK inhibitors, CKIs such as p21 and p27 and the INKs such as p15 and p16 (20–22). These inhibitors play a key role in making sure the cells stop at the R point if any DNA damage is detected. This allows the cells to repair the damage before replicating their DNA. Growth factors such as PDGF and epidermal growth factor activate several pathways, some of which have been shown to directly regulate the cell cycle (22). For example, activation of the Ras/Raf/MEK/ERK kinase cascade, which results in increased expression of cyclin D1 that in turn will activate CDK4/CDK6 allowing cells to traverse the R point. PDGF activation of the Ras/RhoA pathway results in the degradation of p27, which also will have the same overall effect of allowing cells to traverse the R point and enter S phase of the cell cycle (23).

The G<sub>1</sub> arrest brought about by GGTI-298 inhibition of protein geranylgeranylation could be due to effects on several important steps in G<sub>1</sub>/S such as the inhibition of expression of cyclins D and/or E, CDK2, CDK4 and/or CDK6, or increased expression of CKIs and/or INKs. The work described in this study suggests a possible mechanism for GGTI-298 growth

arrest involving increased expression and partner switching of CDK inhibitors, resulting in inhibition of CDK2 and CDK4, and pRb phosphorylation.

#### EXPERIMENTAL PROCEDURES

**Synthesis of CAAX Peptidomimetic**—The GGTase I-specific peptidomimetic GGTI-298 was synthesized as described previously (10, 14).

**Cells and Culture**—Human tumor cell lines Calu-1 and A-549 (lung carcinomas), T-24 (bladder carcinoma), and A-253 (head and neck squamous cell carcinoma) were purchased from ATCC (Manassas, VA) and grown in McCoy's 5A (Calu-1, T-24, and A-253) and F12K (A-549) media at 37 °C in a humidified incubator containing 10% CO<sub>2</sub>.

**Western Blotting**—Cells were treated with GGTI-298 (15  $\mu$ M) for 48 h, harvested, and lysed in HEPES lysis buffer as described previously (11, 14). Proteins were then resolved by 12.5% or 7% SDS-PAGE gel and immunoblotted with antibodies against Rap1A/Krev-1(121), p21<sup>WAF1</sup> (C-190), cyclin E (C-19), cyclin D1 (72-13G), CDK2 (M2), CDK4 (H-22), CDK6 (C-21), p16<sup>INK4A</sup> (C-20), p15<sup>INK4B</sup> (C-20), cyclin A (H-432) (all from Santa Cruz Biotechnology, Santa Cruz, CA), p27<sup>KIP1</sup> (G173-524), and pRb (G3-245) (from Pharmingen, San Diego, CA). The ECL blotting system (NEN Life Science Products) was used for detection of positive antibody reactions (14).

**Flow Cytometry Analysis**—Cells were treated and harvested as described for Western blotting, and nuclei were stained with propidium iodide. DNA content was analyzed by fluorescence-activated cell sorter as described previously (19).

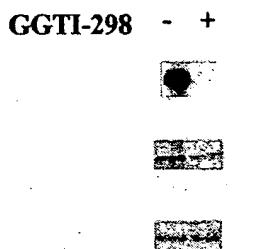
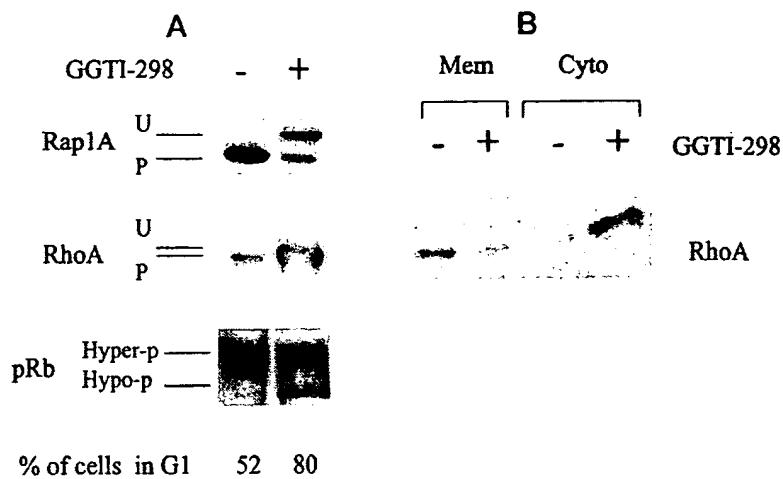
**Immunoprecipitations**—Cells were treated and harvested as described above for Western blotting. Lysates (500  $\mu$ g) were then immunoprecipitated with polyclonal antibodies to CDK2 (M2), CDK4 (H-22), and CDK6 (C-21). The immunoprecipitates were then electrophoresed on a 12.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with p15, p16, p21, and p27 as described above.

**Cyclin-dependent Kinase Assay**—To measure the activity of CDK2, histone H1 was used as the substrate; for CDK4 and CDK6, GST-Rb (C-terminal fragment of pRb) was used as a substrate. The CDK immunoprecipitates were resuspended in 10  $\mu$ l of 50 mM Hepes (pH 7.4) containing 10 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM dithiothreitol, 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and 100  $\mu$ g/ml histone H1 (BM) or 20  $\mu$ g/ml GST-Rb, and then incubated for 30 min at 30 °C with occasional mixing. The reaction was terminated with an equal volume of 2 $\times$  loading buffer (93.75 mM Tris, pH 6.8, 15% glycerol, 3% SDS, 7.5%  $\beta$ -mercaptoethanol). The sample was fractionated by SDS-PAGE, and phosphorylated proteins were visualized by autoradiography.

#### RESULTS

**GGTI-298 Induces Accumulation of Hypophosphorylated pRb in the Human Lung Carcinoma Calu-1**—GGTI-298 was previously shown to inhibit the growth in nude mice and to induce G<sub>1</sub> block of human tumor cells (12, 15). We sought to understand how GGTI-298 prevents Calu-1 cells from traversing G<sub>1</sub> and entering S phase of the cell cycle. We first determined the ability of GGTI-298 to affect phosphorylation of pRb, one of the key events required for G<sub>1</sub>/S transition. Calu-1 cells were treated for 48 h with GGTI-298 (15  $\mu$ M), and the cell lysates were immunoblotted with an anti-pRb antibody that recognizes both hypo- and hyperphosphorylated forms of pRb as described under "Experimental Procedures." Other cell lysate aliquots were immunoblotted with antibodies to either Rap1A or RhoA, small G-proteins that are exclusively geranylgeranylated. Finally, a set of cells were analyzed by flow cytometry to determine the proportion of cells at different phases of the cell cycle. Fig. 1A shows that Calu-1 cells treated with the vehicle contained predominantly hyperphosphorylated pRb. Treatment with GGTI-298 resulted in hypophosphorylation of pRb (Fig. 1A). Hypophosphorylation of pRb correlated with inhibition of the geranylgeranylation of the GGTase I substrates, Rap1A and RhoA, and increased the proportion of Calu-1 cells in the G<sub>1</sub> phase of the cell cycle (Fig. 1A). Because processed and unprocessed RhoA migrated closely (Fig. 1A), we confirmed the effects of GGTI-298 on the processing of RhoA by isolating membranes and cytosolic fractions and showing that GGTI-298 decreases the membrane levels of RhoA while inducing accumulation of RhoA in the cytosol (Fig. 1B).

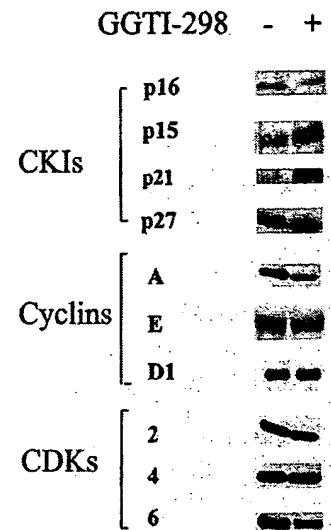
**FIG. 1. GGTI-298 treatment induces pRb hypophosphorylation.** *A*, Calu-1 cells were treated for 48 h with GGTI-298 (15  $\mu$ M), cell lysates were prepared, separated by SDS-PAGE, and immunoblotted with either an anti-pRb1, anti-RhoA, or anti-Rap1A antibody as described under "Experimental Procedures." *U* and *P* designate unprocessed and processed forms of Rap1A. The proportion of Calu-1 cells in  $G_1$  was determined by flow cytometry as described under "Experimental Procedures." *B*, Calu-1 cells were treated as described in *A*, except that membrane (*Mem*) and cytosolic (*cyto*) fractions were isolated before SDS-PAGE immunoblotting with anti-RhoA antibody. Data are representative of five independent experiments except for RhoA (two independent experiments).



**FIG. 2. GGTI-298 treatment results in inhibition of the kinase activities of CDK2 and CDK4.** Calu-1 cells were treated with GGTI-298, cell lysates were prepared and immunoprecipitated with CDK2, CDK4, and CDK6 antibodies, and kinase assays were carried out as described under "Experimental Procedures." Data are representative of at least three independent experiments.

**GGTI-298 Inhibits the Kinase Activities of CDK2 and CDK4**—We next evaluated the ability of GGTI-298 to inhibit  $G_1$  phase cyclin-dependent kinases that phosphorylate pRb. Calu-1 cells were treated with GGTI-298 for 48 h, and the lysates immunoprecipitated with anti-CDK2, anti-CDK4, or anti-CDK6 antibody as described under "Experimental Procedures." Fig. 2 shows that CDK2, CDK4, and CDK6 from control Calu-1 cells were active and phosphorylated histone H1 (CDK2) and GST-Rb (CDK4 and CDK6) *in vitro*. Treatment with GGTI-298 blocked the activity of CDK2, inhibited CDK4 and CDK6 activities by 75% and 30%, respectively (Fig. 2).

**Effects of GGTI-298 on the Expression of Various Cell Cycle Components**—The mechanism by which GGTI-298 results in inhibition of the activities of CDKs could involve inhibition of the expression of the  $G_1$  kinases themselves or their cyclins A, D, and E, or because of an increase in the levels of cyclin-dependent kinase inhibitors. To further investigate these possibilities, Calu-1 cells were treated with GGTI-298 for 48 h, and the lysates immunoblotted with antibodies to CDK2, CDK4, CDK6, cyclins D1, E, and A, p21, p27, p15, and p16 as described under "Experimental Procedures." Fig. 3 shows that control-dividing Calu-1 cells expressed all cell cycle components evaluated except p21, which was barely detectable. Treatment of Calu-1 cells with GGTI-298 did not alter the levels of CDK2 and CDK4 or those of cyclins E and D1, indicating that the inhibition of CDK2 and CDK4 activities is not because of a decrease in the levels of cyclins E and D1 or the kinases (Fig. 3). However, the amount of cyclin A was decreased by 20%, and this could contribute to the block of CDK2 kinase activity. The levels of CDK6 decreased by 15%, and this could account for the decrease in its kinase activity (Fig. 2). Furthermore GGTI-298 greatly increased (7-fold) the levels of p21, and to a lesser extent (2-fold) increased the levels of p15 and had little effect



**FIG. 3. Effects of GGTI-298 on the protein levels of CKIs, cyclins, and CDKs.** Calu-1 cells were treated and processed as described in the legend for Fig. 1, except lysates were immunoblotted with antibodies to the indicated proteins as described under "Experimental Procedures." Data are representative of at least six independent experiments.

on the levels of p16 and p27 (Fig. 3).

**GGTI-298 Induces Partner Switching of CDK Inhibitors**—The above data suggest that the inhibition of CDK2 and CDK4 and the subsequent hypophosphorylation of pRb are caused by increased protein levels of some of the CDK inhibitors. Therefore we investigated whether association of the CDKs with their inhibitors was also affected. Calu-1 cells were treated with vehicle (control) or GGTI-298 for 48 h, the lysates were immunoprecipitated with various CDKs, the immunoprecipitates were separated by SDS-PAGE and immunoblotted by various CKIs. Fig. 4 shows that in control cells, p21 and p15 were associated with primarily CDK6, whereas p27 was primarily associated with CDK4 and CDK6. Treatment with GGTI-298 increased (7-fold) association of p21 with CDK2 while decreasing (70%) its association with CDK6. Similarly, GGTI-298 treatment decreased binding of p27 to CDK6 (85%) and CDK4 (22%) and increased binding to CDK2 (2-fold) (Fig. 4). Furthermore, GGTI-298 treatment increased (2-fold) association of p15 to CDK4 with no effects on association with CDK6 (Fig. 4). Finally, GGTI-298 had no effect on the association of p16 with CDK4 or CDK6 (data not shown). Thus, GGTI-298 treatment resulted in a partner switch for p21 and p27 from CDK6 to CDK2. Furthermore, GGTI-298 treatment

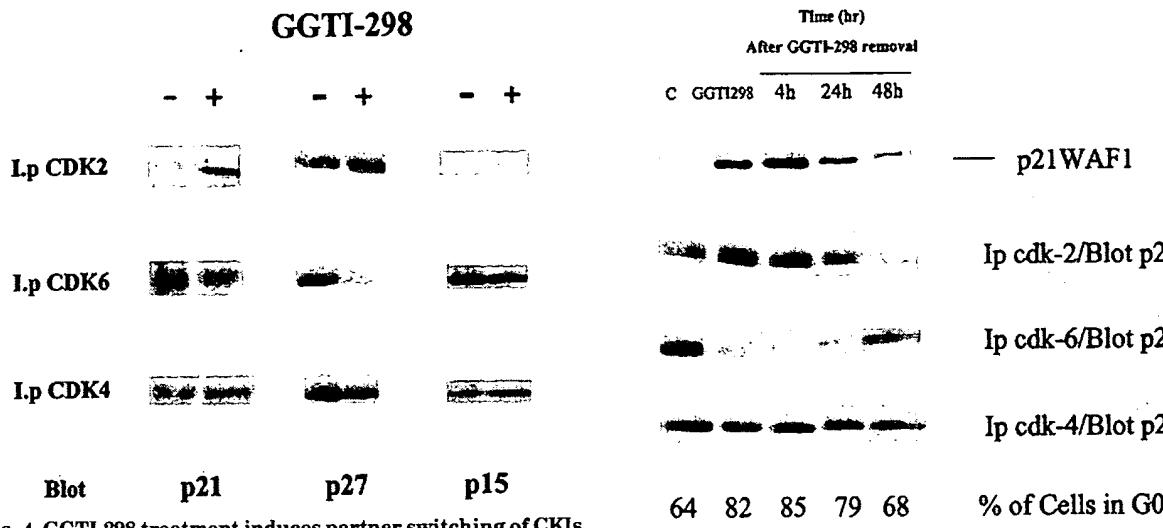


FIG. 4. GGTI-298 treatment induces partner switching of CKIs. Calu-1 cells were treated and processed as described in the legend to Fig. 2, except the immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies for p21, p27, and p15. Data are representative of at least four independent experiments.

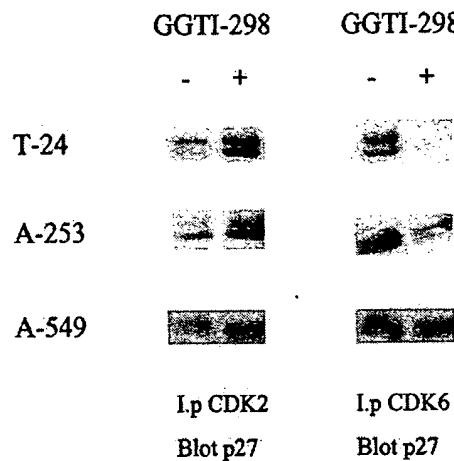


FIG. 5. GGTI-298 induces partner switching in several human tumor cell lines. A-549, T-24, and A-253 cells were treated and processed as described in the legend to Fig. 4. Data are representative of two independent experiments.

also induced an increased association of CDK4 with p15 while decreasing its association with p27.

We next determined whether the GGTI-298-induced partner switching is unique to Calu-1 cells. To this end, we treated three other human tumor cell lines; A-549 lung carcinoma, T-24 bladder carcinoma, and A-253 head and neck squamous cell carcinoma with GGTI-298, immunoprecipitated the lysates with CDK2 and CDK6 antibodies, and blotted with p27 antibody as described under "Experimental Procedures." Fig. 5 shows that in all three cell lines treatment with GGTI-298 increased the levels of p27 associated with CDK2 while it decreased the levels associated with CDK6.

**Removal of GGTI-298 Results in Reversal of Partner Switching**—To determine whether removal of GGTI-298 would result in reversal of G<sub>1</sub> block and partner switching, we treated Calu-1 cells with GGTI-298 for 48 h, fresh medium lacking GGTI-298 was then added, and the cells collected after 0, 4, 24, and 48 h post-GGTI-298 removal. The cells were then harvested and processed for p21<sup>waf</sup> induction, immunoprecipitation/blotting and flow cytometry as described under "Experimental Procedures." Fig. 6 shows that, consistent with Fig. 4,

FIG. 6. Time course reversal of GGTI-298 effects. Calu-1 cells were treated with either vehicle (control) or with GGTI-298 for 48 h at which time fresh medium lacking GGTI-298 was added, and the cells were collected at 0, 4, 24, and 48 h. The cells were then processed for p21<sup>waf</sup> immunoblotting, flow cytometry, and immunoprecipitation with CDK2, CDK4, and CDK6 antibodies, followed by immunoblotting with p27 antibody as described under "Experimental Procedures." Data are representative of two independent experiments.

GGTI-298 induced p21<sup>waf</sup>, partner switching of p27 from CDK6 to CDK2 and G<sub>1</sub> block (compare control versus GGTI-298 lanes of Fig. 6). GGTI-298 had little effect on the association of p27 with CDK4. Removal of GGTI-298 resulted in a time-dependent reversal of p21<sup>waf</sup> induction, partner switching, and G<sub>1</sub> block. Little change was observed 4 h after removal. However, significant reversal occurred at 48 h (Fig. 6).

#### DISCUSSION

Although there are more geranylgeranylated proteins than farnesylated proteins, more efforts have been spent on designing, synthesizing, and biologically characterizing FTase rather than GGTase I inhibitors (8). The intense search for FTase inhibitors was prompted some years ago by the realization that farnesylation is required for the cancer-causing activity of the important oncoprotein Ras (8). Recently, however, more attention has been directed toward understanding the effects of inhibiting (by GGTase I inhibitors) the function of geranylgeranylated proteins. This is primarily due to the discovery that some geranylgeranylated proteins such as those from the Rho family are essential for normal and aberrant proliferation in several cell types (1, 5). In addition to their ability to inhibit human tumor growth, GGTase I inhibitors may also have great therapeutic potential in cardiovascular diseases. For example, GGTI-298 blocks the ability of smooth muscle cells to proliferate by inducing a G<sub>1</sub> block and apoptosis (16). This may be related to the inhibition by GGTI-298 of PDGF and Ras induction of superoxide formation (18) suggesting that a geranylgeranylated protein downstream of Ras is critical to events regulating cell division. This is consistent with a recent report, which shows that the geranylgeranylated protein Rac1 mediates superoxide formation and transformation by Ras (24). In this study, we demonstrate that GGTI-298 treatment of the human lung carcinoma Calu-1 cells results in a large increase of the CDK inhibitor p21 and a modest increase of p15 with little effect on p27 and p16. In nontreated dividing cells, CDK2 and CDK4 bound mainly p27 and their kinases were active, whereas CDK6 bound all inhibitors and was also active. Upon treatment with GGTI-298, p21 and p27 switched partners from CDK6 to CDK2, whereas p15 became bound to CDK4. The

GGTI-298 treatment of the human lung carcinoma Calu-1 cells results in a large increase of the CDK inhibitor p21 and a modest increase of p15 with little effect on p27 and p16. In nontreated dividing cells, CDK2 and CDK4 bound mainly p27 and their kinases were active, whereas CDK6 bound all inhibitors and was also active. Upon treatment with GGTI-298, p21 and p27 switched partners from CDK6 to CDK2, whereas p15 became bound to CDK4. The

effects of GGTI-298 on the observed partner switching was reversible. We found that removal of GGTI-298 resulted in reversal of the G<sub>1</sub> phase block, which was paralleled by a reversal of partner switching of p27 from CDK2 to CDK6.

The fact that in dividing cells, CDKs are bound to some inhibitors is not surprising, because low levels of such inhibitors may be required to stabilize cyclins with the corresponding CDKs (25). This is also consistent with the suggestion that some CDKs may serve as cellular reservoirs for low levels of inhibitors (26). Furthermore, although there was little increase in total cellular levels of p27 after GGTI-298 treatment, the amount of p27 bound to CDK2 was increased. This suggests that p27 that was released from CDK6, and to a lesser degree from CDK4, bound to CDK2. Taken altogether, the data suggests that GGTI-298 induced partner switching for p21 and p27 from CDK6 to CDK2. This partner switching was not unique to Calu-1 cells and was found to occur in three other human tumor cell lines, A-549, T-24, and A-253. These results are similar to those obtained from cells that were arrested in late G<sub>1</sub> following treatment with transforming growth factor  $\beta$  (27). In these cells, transforming growth factor  $\beta$  induced the expression of p15, which bound CDK4 and CDK6 and displaced p27 from cyclin D-CDK4 and cyclin D-CDK6 complexes (27). The free p27 bound the cyclin E-CDK2 complex and inhibited its activity, which resulted in preventing cells from entering S phase (27).

The fact that inhibition of protein geranylgeranylation results in G<sub>1</sub> arrest suggests that some geranylgeranylated proteins are required for cell progression from G<sub>1</sub> to S. The most likely candidates are members of the Rho family of proteins such as RhoA and Rac1. In this study, we showed that GGTI-298 inhibits the processing of RhoA, and recently we have demonstrated that RhoA represses p21<sup>waf</sup> transcription and suggested that GGTI-298 induction of p21<sup>waf</sup> is mediated by inhibition of RhoA geranylgeranylation (29). Furthermore, it has been shown that both RhoA and Rac1, when microinjected into G<sub>1</sub>-arrested cells, promote progression to S phase (5). Furthermore, dominant-negative Rac1 and RhoA reverse oncogenic Ras transformation (6, 7). Rac1 may regulate transcription by activating the Jun kinase pathway that in turn will affect genes that are regulated by AP-1. Furthermore, very recently Tapon *et al.* (28) discovered a novel effector of Rac1, POSH, a kinase that appears to be required for activation of Jun kinase by Rac1. It would be of great interest to determine whether activated Jun kinase or POSH would rescue the cells

from the GGTI-298 mediated G<sub>1</sub> block. These studies, which are in progress, will further enhance our understanding of how inhibition of protein geranylgeranylation results in G<sub>1</sub> arrest.

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# Calmodulin Is Essential for Cyclin-dependent Kinase 4 (Cdk4) Activity and Nuclear Accumulation of Cyclin D1-Cdk4 during G<sub>1</sub>\*

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Although it is known that calmodulin is involved in G<sub>1</sub> progression, the calmodulin-dependent G<sub>1</sub> events are not well understood. We have analyzed here the role of calmodulin in the activity, the expression, and the intracellular location of proteins involved in G<sub>1</sub> progression. The addition of anti-calmodulin drugs to normal rat kidney cells in early G<sub>1</sub> inhibited cyclin-dependent kinase 4 (Cdk4) and Cdk2 activities, as well as retinoblastoma protein phosphorylation. Protein levels of cdk4, cyclin D1, cyclin D2, cyclin E, p21, and p27 were not affected after CaM inhibition, whereas decreases in the amount of cyclin A and Cdc2 were observed. The decrease of Cdk4 activity was due neither to changes in its association to cyclin D1 nor to changes in the amount of p21 or p27 bound to cyclin D1-Cdk4 complexes. Calmodulin inhibition also produced a translocation of nuclear cyclin D1 and Cdk4 to the cytoplasm. This translocation could be responsible for the decreased Cdk4 activity upon calmodulin inhibition. Immunoprecipitation, calmodulin affinity chromatography, and direct binding experiments indicated that calmodulin associates with Cdk4 and cyclin D1 through a calmodulin-binding protein. The facts that Hsp90 interacts with Cdk4 and that its inhibition induced Cdk4 and cyclin D1 translocation to the cytoplasm point to Hsp90 as a good candidate for being the calmodulin-binding protein involved in the nuclear accumulation of Cdk4 and cyclin D1.

Calmodulin (CaM)<sup>1</sup> is the main Ca<sup>2+</sup>-binding protein in non-muscle cells. The functions of CaM are mediated by its association with specific target proteins, *i.e.* CaM-binding proteins (CaMBPs). CaMBPs include a great variety of proteins, some of which are located in the cell nucleus (*e.g.* kinases, such as

CaM-dependent protein kinases II and IV and myosin light chain kinase, phosphatases, such as calcineurin, RNA-binding proteins, such as hnRNP A2 and hnRNP C, and the autoantigen La/SSB), indicating that CaM regulates nuclear functions (1).

It is now well established that CaM is involved in the regulation of the cell cycle (2). By using expression vectors capable of inducibly synthesizing CaM sense or antisense mRNAs, it has been shown that progression through G<sub>1</sub> and mitosis exit is sensitive to changes in the intracellular concentration of CaM (3). Furthermore, the addition of specific anti-CaM drugs to cell cultures inhibits reentry of growth-arrested cells into the cell cycle (G<sub>0</sub>/G<sub>1</sub> transition), progression into and through the S phase, and entry and exit from mitosis (4–10).

Recent reports also give some suggestions of the role of CaM on G<sub>1</sub>/S transition, although some of the results are controversial. The addition of anti-CaM drugs to NRK cells during the early G<sub>1</sub> inhibits the onset of DNA synthesis (9). Inhibition of the CaMKII also blocks G<sub>1</sub> progression in NIH 3T3 cells (11). On the contrary, calcineurin, but not CaMKII, has been shown to be essential for G<sub>1</sub>/S transition in Swiss 3T3 cells (12). The activation or expression of several enzymes and proteins involved in DNA replication, such as DNA polymerases  $\alpha$  and  $\delta$  and the proliferating cell nuclear antigen, is inhibited after the addition of anti-CaM drugs during early G<sub>1</sub> (9, 13, 14). Furthermore, CaM has also been shown to be essential for retinoblastoma protein (pRb) phosphorylation (15). All of these results suggest that CaM is essential for the activation of the cell cycle regulatory machinery involved in progression from G<sub>1</sub> to S phase.

In mammalian cells, progression through the cell cycle is regulated by a family of serine/threonine protein kinases called cyclin-dependent kinases (Cdks). These kinases are formed by two subunits, a catalytic subunit that is present throughout the cell cycle and a regulatory subunit, called cyclin, that is present only at specific stages of the cell cycle. Cyclin D-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2, and cyclin B-Cdk1 are sequentially activated during G<sub>1</sub>, G<sub>1</sub>/S transition, S phase, and mitosis, respectively (16–19).

The activity of the cdks is regulated by several mechanisms: cyclin binding, phosphorylation, and binding to specific Cdk-inhibitory proteins (20, 21). Activating and inhibitory phosphorylation sites exist in the catalytic subunit (22–24). To date, seven Cdk-inhibitory proteins have been identified in mammalian cells (21). They are divided into two major families: INK4 and CIP/KIP. The INK4 family includes p16, p15, p18, and p19, which interact specifically with Cdk4 and Cdk6. Addition of p16 to cyclin D-Cdk4 complexes results in the dissociation of the kinase complex, and binding of p16 to monomeric Cdk4 prevents cyclin D association (25). The CIP/KIP family of inhibitors comprises three members: p21, p27, and p57. They

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<sup>1</sup> The abbreviations used are: CaM, calmodulin; CaMBP, calmodulin-binding proteins; NRK, normal rat kidney; CaMKII, CaM-dependent kinase II; Cdk, cyclin-dependent kinase; FCS, fetal calf serum; W12, N-(4-aminobutyl)-2-naphthalenesulfonamide; W13, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; W7, N-(6-aminohexyl)-5-iodo-1-naphthalenesulfonamide; J8, N-(8-aminoctyl)-5-iodo-1-naphthalenesulfonamide; PBS, phosphate-buffered saline; BrdUrd, 5-bromo-deoxyuridine; NLS, nuclear location sequence; Hsp, heat shock protein; pRb, retinoblastoma protein; TBS, Tris-buffered saline; GST, glutathione S-transferase.

have a broad range of specificity and can inhibit all of the G<sub>1</sub> cdk-cyclin complexes. Moreover, they have higher affinity for the cyclin-Cdk complexes than for the monomeric cdk subunit (26, 27).

The major function of cyclin D-Cdk4 during G<sub>1</sub> is the phosphorylation of the pRb family members (28, 29). The transcription factor E2F is bound to the hypophosphorylated form of pRb, but after phosphorylation of pRb, E2F is released, and then it activates transcription of S-phase-specific genes (30, 31). Among the genes containing E2F-binding sites in their promoter regions are those encoding for enzymes and proteins that are essential for DNA replication, such as DNA polymerase  $\alpha$ , thymidine kinase, ribonucleotide reductase, cyclin A, and cyclin E (32–36). The activation of cyclin D1-Cdk4 and phosphorylation of pRb is thus a limiting step for G<sub>1</sub> progression. Cdk2-cyclin E is also essential for G<sub>1</sub>/S transition. It also phosphorylates pRb and possibly some other proteins essential for the onset of DNA replication (18, 37).

Despite the evidence indicating that CaM plays a role in G<sub>1</sub> progression, not much is known about the specific mechanisms regulated by CaM during this phase. Here, we analyzed the effect of the addition of an anti-CaM drug during G<sub>1</sub> on the activation and regulation of G<sub>1</sub> cyclin-cdk complexes.

#### EXPERIMENTAL PROCEDURES

**Cell Cultures**—NRK cells were made quiescent by growing them to confluence in Dulbecco's minimum essential medium supplemented with 5% FCS and then kept for 3 days in the same medium but with only 0.5% FCS. To allow them to reenter the cell cycle, quiescent cells were trypsinized and subcultured at a lower density in fresh medium supplemented with 5% FCS. Unless otherwise indicated, the anti-CaM drug W13 or the control drug W12 was added to the cell culture medium at a final concentration of 15  $\mu$ g/ml at 5 h after proliferative activation of the cells. Other anti-CaM drugs used were W7, J8, and calmidazolium. They were also added 5 h after proliferative activation. *In vivo* DNA synthesis was examined by measuring the incorporation of [<sup>3</sup>H]thymidine into whole cell DNA. NRK cells (10<sup>6</sup> per 35-mm plate) were pulse-labeled for 1 h with [*methyl-<sup>3</sup>H*]thymidine (5 Ci/mmol) (Amersham Pharmacia Biotech) at 4  $\mu$ Ci/ml in medium supplemented with 5% FCS. Precipitation and solubilization of DNA were done as described previously (9).

**Gel Electrophoresis and Immunoblotting**—Cells were lysed in a buffer containing 2% SDS, 67 mM Tris-HCl, pH 6.8, and 10 mM EDTA. The extracts were electrophoresed on SDS-polyacrylamide gels essentially as described in Ref. 38. Two-dimensional gels were run as described in Ref. 39. After electrophoresis, the proteins were transferred to Immobilon-P strips for 2 h at 60 V, for 20 h at 30 V in the case of pRb analysis, or for 1 h at 60 V in the case of CaM analysis. The sheets were preincubated in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), 0.05% Tween 20, and 5% bovine serum albumin for 1 h at room temperature and then incubated for 1 h at room temperature in TBS, 0.05% Tween 20, 1% bovine serum albumin, and 0.5% nonfat milk powder containing antibodies against Cdk2 (06-505, Upstate Biotechnology, 3  $\mu$ g/ml), Cdc2 (06-194, Upstate Biotechnology, 3  $\mu$ g/ml), Cdk4 (sc-260-R, Santa Cruz Biotechnology, 0.5  $\mu$ g/ml), cyclin A (06-138, Upstate Biotechnology, 7  $\mu$ g/ml), cyclin E (sc-198, Santa Cruz Biotechnology, 1  $\mu$ g/ml), cyclin D (06-450, Upstate Biotechnology, 2  $\mu$ g/ml), pRb (sc-50, Santa Cruz Biotechnology, 2  $\mu$ g/ml), p21 (sc-397, Santa Cruz Biotechnology, 2  $\mu$ g/ml), and p27 (1:500 dilution; gift from Dr. Massagué, New York, NY) polyclonal antibodies or CaM (06-45005-173, Upstate Biotechnology, 1:2000 dilution) monoclonal antibody. After washing in TBS, 0.05% Tween 20 (three times, 10 min each), the sheets were incubated with either a peroxidase-coupled secondary antibody (1:1000 dilution) (Bio-Rad) or an alkaline phosphatase-coupled secondary antibody (1:10000) (Promega) for 1 h at room temperature. After incubation, the sheets were washed twice in TBS, 0.05% Tween 20 and once in TBS. The reaction was visualized by ECL (Amersham Pharmacia Biotech) or with BCIP/NBT (Promega).

**Immunoprecipitation and Kinase Assays**—To detect proteins associated with cdk4, immunoprecipitations were performed as described in Ref. 40. Cells (5–10  $\times$  10<sup>7</sup>) were lysed in 1 ml of Buffer A (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml leupeptin). 3–5 mg of protein from the lysates were incubated

with 4  $\mu$ g of anti-Cdk4 (sc-260-R, Santa Cruz Biotechnology) or anti-cyclin D1 (06-137, Upstate Biotechnology) antibodies or with 3  $\mu$ l of normal rabbit serum (controls) for 2 h at 4 °C. Protein immunocomplexes were then incubated with 40  $\mu$ l of protein A-Sepharose (Pierce) for 1 h at 4 °C, collected by centrifugation, and washed three times in Buffer A. Immunoprecipitated proteins were then analyzed by electrophoresis and Western blotting. A lysate from NRK cells was always loaded in the same gel in order to have a control for the mobility of each protein. To analyze coimmunoprecipitation of CaM with Cdk4 or cyclin D1, cells were lysed in Buffer B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml leupeptin) and then incubated with the anti-Cdk4 antibodies as indicated above. In this case, the protein A-Sepharose used was previously blocked with a solution of bovine serum albumin (2 mg/ml). For kinase assays, immunoprecipitations were performed similarly, but 5  $\times$  10<sup>6</sup> cells were used per assay, and the lysates were first precleared by addition of 30  $\mu$ l of normal rabbit serum and 50  $\mu$ l of protein A-Sepharose. The immunoprecipitated complexes were washed in kinase buffer (HEPES-Na, pH 7.4, 10 mM magnesium acetate, and 1 mM dithiothreitol) and then incubated in kinase buffer containing 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (2–4  $\times$  10<sup>4</sup> cpm/pmol) and 2.5  $\mu$ g of histone H1 (for Cdk2) or 1  $\mu$ g of PGST-Rb (379–792) fusion protein (for Cdk4 kinase) for 20 min at 30 °C in a final volume of 35  $\mu$ l (41). Then, the samples were electrophoresed on SDS-polyacrylamide gels, and the gels were stained with Coomassie Blue, dried, and exposed to x-ray films at –80 °C. GST-Rb (379–792) fusion protein expression plasmid was a kind gift from Dr. Graña (Philadelphia, PA).

**Immunocytochemistry**—Quiescent cells were grown on glass coverslips. To detect Cdk4 or cyclin D, cells were fixed in cold methanol for 2 min. For nucleolin staining, cells were fixed in 3% paraformaldehyde/PBS (140 mM NaCl, 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. In all cases, the nonspecific sites were subsequently blocked with 1% ovalbumin/PBS for 10 min at room temperature. Cells were then incubated 1 h at 37 °C in a humidified atmosphere, with the specific polyclonal antibodies anti-Cdk4 (sc-260-R, Santa Cruz Biotechnology, 1:50 dilution; C-18720, Transduction Laboratories 1:20 dilution), anti-cyclin D1 (06-194, Upstate Biotechnology, 1:50 dilution) or anti-nucleolin (1:200 dilution; a gift of Dr. Ghisolfi-Nieto). Coverslips were then washed three times (5 min each) in PBS and incubated for 45 min at 37 °C with fluorescein-conjugated anti-rabbit antibody (1:100 dilution, Sigma). After two washes in PBS, coverslips were mounted on glass slides with Mowiol (Ciba-Geigy). The specificity of the antibody response was demonstrated by the preabsorption of the antibodies with the antigenic peptide. In order to quantify nuclear Cdk4 or cyclin D1 after anti-CaM drugs treatment, peroxidase-conjugated secondary antibodies were used and visualized developing with 3,3'-diaminobenzidine. The ratio of absorbance to area was obtained using KS 100 Kontron Imaging System software. Nuclei were considered stained when the ratio of absorbance to area was above 0.1.

**Microinjection**—Quiescent cells were seeded onto glass coverslips at 50–60% confluence and activated as described above. Coverslips were transferred into Dulbecco's minimum essential medium (HEPES modification) (Sigma) 5% FCS fresh medium just before injection, (5–8 h after proliferative activation). The CaM-dependent protein kinase II fragment 290–309 (42) aqueous solution was mixed with molecular weight 155,000 rhodamine-dextran (Sigma) in PBS, to act as an injection marker (final concentrations, 240  $\mu$ M peptide and 1% dextran). Microinjection of 1% dextran in PBS solution was used as a control. Cytoplasmic injections were carried out with a Zeiss automated injection system equipped with an Eppendorf 5246 transsector. After injection coverslips were placed into fresh Dulbecco's minimum essential medium 5% FCS medium and incubated for 1 h before fixation and immunocytochemical processing as described above to measure nuclear staining of Cdk4. To measure the labeling index with BrdUrd, coverslips were incubated until 21 h after proliferative activation, BrdUrd (3  $\mu$ g/ml) was added to the medium at 19 h, and cells were fixed in ethanol/acetic acid (95:5) for 30 min. BrdUrd was detected with monoclonal anti-bromodeoxyuridine antibody (Amersham Pharmacia Biotech) according to the manufacturer's procedures using fluorescein-conjugated anti-mouse antibody (dilution 1:100, Boehringer Mannheim) as secondary antibodies. Microinjected cells were detected by the rhodamine cytoplasmic signal, and BrdUrd or cdk4 nuclear staining was detected by the fluorescein signal. Quantitation of nuclear cdk4 staining was performed with the KS 100 Kontron Imaging System software. The “meangreen” (mean densitometric value of channel green) of each nucleus was recorded. Nuclei were considered stained when meangreen was above 100 grey units. The results represent the average of at least

two separate experiments. Minimum totals of 80 and 40 cells were measured for peptide/dextran- and dextran-injected cells, respectively.

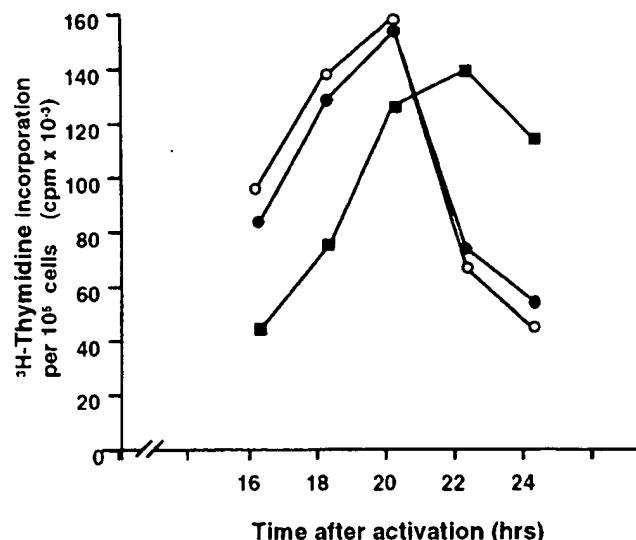
**Calmodulin-Sepharose Affinity Chromatography**—Cells ( $2 \times 10^7$ ) were lysed in 500  $\mu$ l of Buffer C (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 200  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM phenylmethylsulfonyl fluoride, 1.5  $\mu$ M pepstatin, 2  $\mu$ g/ml leupeptin, 4  $\mu$ g/ml aprotinin) containing 5 mM EGTA. Solubilized proteins (1 mg) were loaded to a 300- $\mu$ l DEAE-Sepharose column preequilibrated with Buffer C. Proteins not bound to the column were collected. Under these conditions, endogenous CaM remains bound to the column, whereas Cdk4 is found in the flow-through fraction. An adequate volume of 100 mM CaCl<sub>2</sub> was added to this fraction to obtain a final concentration of 7 mM CaCl<sub>2</sub> and then was mixed with 300  $\mu$ l of CaM-Sepharose 4B preequilibrated with Buffer C containing 2 mM CaCl<sub>2</sub>. The resin was subsequently washed with the same buffer, and finally, the CaMBPs were eluted three times with 300  $\mu$ l of Buffer C containing 5 mM EGTA. Eluted fractions were subsequently analyzed by Western blot. For the pull-down experiments, flow-through from the DEAE-Sepharose (containing 5 mM EGTA) was incubated for 2 h at 4 °C with 40  $\mu$ l of CaM-Sepharose or Sepharose alone in the same buffer (5 mM EGTA) or after the addition of 7 mM Ca<sub>2</sub>Cl. Samples were washed five times with lysis buffer C containing either EGTA or free Ca<sup>2+</sup> and then extracted directly with SDS-polyacrylamide gel electrophoresis Laemmli loading buffer and analyzed by Western blot.

## RESULTS

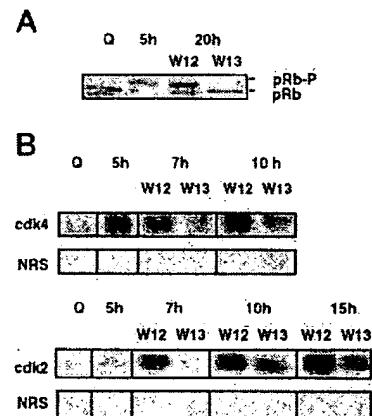
**Inhibition of CaM during Early G<sub>1</sub> Blocked pRb Phosphorylation and Cdk4 and Cdk2 Activation**—When NRK cells were activated to proliferate from quiescence, they reenter the cell cycle and start DNA synthesis at 12 h, reaching a maximum at 18–20 h (9, 43). Addition of different anti-CaM drugs 5 h after activation (first half of G<sub>1</sub>) inhibited the entrance of the cells into S phase, the activation of DNA polymerases  $\alpha$  and  $\delta$ , and the expression of proliferating cell nuclear antigen (9, 13, 14). Addition of the same anti-CaM drugs later in G<sub>1</sub> had much less effect. We first analyzed whether this inhibitory effect was reversible. The anti-CaM drug used was W13. W12 was used as a control because it is chemically very similar to W13 but it has much lower affinity for CaM. W13 has been extensively used to inhibit CaM in cell cultures, and it is known to be highly specific at the dosages used in this work (6, 9, 13, 14). W13 (15  $\mu$ g/ml) or W12 (15  $\mu$ g/ml) was added to the medium 5 h after proliferative activation of the cells. Drugs were removed 2 h later, and the cells were incubated with fresh media containing 5% FCS. Thymidine incorporation during 1 h was measured in nontreated and W13- and W12-treated cells at 18 h, 20 h, 22 h, and 24 h. Nontreated and W12-treated cells showed a maximum of thymidine incorporation at 20 h, whereas W13-treated cells reached the same level of thymidine incorporation but with a maximum at 22 h (Fig. 1). Thus, inhibition of CaM for 2 h at mid G<sub>1</sub> produced a reversible G<sub>1</sub> arrest, and cells synchronously proceed into S phase upon removal of W13 with a delay of 2 h. One of the key events for G<sub>1</sub> to S phase progression is Cdk4 and Cdk2 activation and consequently pRb hyperphosphorylation. Thus, the phosphorylation status of this protein upon anti-CaM drug addition was analyzed. The drug was always added 5 h after proliferative activation of the cells. Thus, they were added after the peak of Fos expression (data not shown).

Hyperphosphorylated pRb has a reduced mobility in SDS-polyacrylamide gels; thus, it can easily be distinguished from the hypophosphorylated form (44). In quiescent cells, pRb was hypophosphorylated. At 5 h, the phosphorylated form was already detected, and at 20 h, in cells treated with control drug (W12), almost all pRb was hyperphosphorylated (Fig. 2A). In W13-treated cells analyzed at 20 h, the hypophosphorylated form was the most abundant, indicating that the inhibition of CaM blocked the phosphorylation of pRb.

To analyze whether the blockage of pRb phosphorylation by CaM inhibitors was due to the inhibition of Cdk4 or Cdk2, their

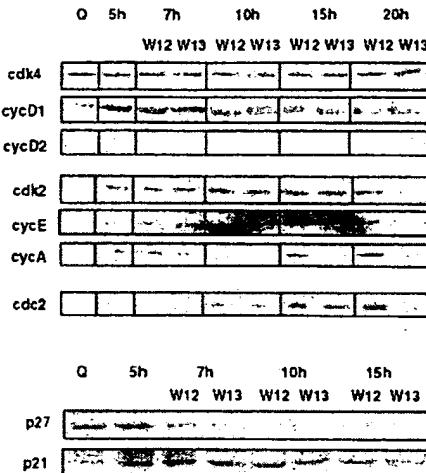


**FIG. 1. Reversible G<sub>1</sub> arrest induced by CaM inhibition.** [<sup>3</sup>H]Thymidine incorporation for 1 h into whole cell DNA was measured at 16, 18, 20, 22, and 24 h after proliferative activation of NRK cells as indicated under “Experimental Procedures.” Open circles are [<sup>3</sup>H]thymidine incorporation into cells that had not been treated with any drug. W12 (closed circles) or W13 (closed squares) was added to the medium (15  $\mu$ g/ml) 5 h after proliferative activation; 2 h later, they were removed, and medium (5% FCS) free of drugs was added.



**FIG. 2. Effect of anti-CaM drug addition on pRb phosphorylation and Cdk4 activity.** **A**, effect of anti-CaM drug treatment on pRb phosphorylation. Quiescent NRK cells (Q), or cells activated to proliferate were lysed at different times (5 and 20 h) after serum addition. Cells harvested at 20 h were treated at 5 h after activation with W12 or W13 (15  $\mu$ g/ml). 50  $\mu$ g of proteins from the lysates were separated on an 6% SDS-polyacrylamide gel, electroblotted, and subjected to Western blot as indicated under “Experimental Procedures.” The antibody recognized the hyperphosphorylated (pRb-P) and the hypophosphorylated (pRb) forms of pRb. **B**, effect of anti-CaM drugs on the activity of Cdk4. Quiescent (Q) NRK cells were activated to proliferate, and at the times indicated in the figure, they were harvested and lysed. The cells harvested at 7, 10, or 15 h were treated with 15  $\mu$ g/ml of W12 or W13 at 5 h after serum addition. Cell lysates were immunoprecipitated with antibodies against Cdk4 or Cdk2. Immunoprecipitations using normal rabbit serum (NRS) were performed as controls. After immunoprecipitation, the kinase activity was analyzed as described under “Experimental Procedures.” To analyze the activity of Cdk4, a fragment of pRb was used as substrate, whereas to detect Cdk2 activity, the substrate used was histone H1.

activities upon W13 addition were measured. Quiescent cells did not show any Cdk4 or Cdk2 activity (Fig. 2B). Cdk4 was already activated 5 h after proliferative stimulation, and its activity was maintained high during G<sub>1</sub> (up to 10 h). Cdk2 was activated later than Cdk4. Its activity was low at 5 h, increased at 7 h, and was high at 15 h (S phase). The activities of both Cdk4 and Cdk2 in W13-treated cells were much lower than in



**FIG. 3. Effect of anti-CaM drug treatment on the levels of Cdk4 and cyclins.** Quiescent (Q) cells were activated to proliferate by serum addition and, at the indicated times, lysed and subjected to Western blot analysis as indicated under "Experimental Procedures." Where indicated, W12 or W13 (15 μg/ml) was added 5 h after serum addition. 30 μg of proteins from the lysates were separated on SDS-polyacrylamide gels and transferred onto membranes as described under "Experimental Procedures." The blots were then incubated with the antibodies against the proteins indicated in the figure. For Cdk4, Cdk2, cyclin D1, and cyclin D2 (cycD1 and cycD2) analysis, the proteins were resolved on 10% SDS-polyacrylamide gels; for the detection of cyclin E and cyclin A (cycE and cycA), the proteins were separated on 8% SDS-polyacrylamide gels; and for p21 and p27, the proteins were separated on 12% SDS-polyacrylamide gels.

control cells at all the times studied (Fig. 2B).

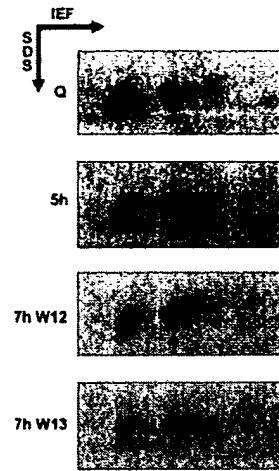
**Effect of CaM Inhibition on the Levels of Cyclins and Cdk4s**—To study how CaM could regulate the activities of Cdk4s, we first measured the amount of cyclins and Cdk4s by Western blot analysis in extracts from quiescent and proliferating cells treated with either W12 or W13. Cdk4 and Cdk2 were detected in quiescent cells. After proliferative activation, the amount of Cdk4 was unchanged, whereas that of Cdk2 increased at 5 h and then remained constant until 20 h. The addition of W13 to the cultures at 5 h did not affect the amount of either Cdk4 or Cdk2 (Fig. 3).

We further analyzed the levels of the different G<sub>1</sub> cyclins in W13-treated cells. As shown in other cellular types, the levels of cyclins D1 and D2 were low, although detectable, in quiescent cells (45, 46). After serum addition, the amount of both cyclins increased slightly, showing a faint peak at 5–7 h. Cyclin D3 was not detected in these cells by Western blot analysis. Addition of W13 to the cultures at 5 h after activation did not have any effect on the amount of any of the D type cyclins (Fig. 3).

Cyclins E and A were not detectable in quiescent cells. Maximal levels of cyclin E were observed at 10–15 h, corresponding to G<sub>1</sub>-S transition, whereas cyclin A showed a large increase at 15–20 h. The treatment with W13 did not affect the amount of cyclin E, whereas the amount of cyclin A was clearly decreased (Fig. 3).

Thus, the addition of W13 to cultures at 5 h after activation induced a decrease of the amount of cyclin A, whereas the levels of Cdk4, Cdk2, cyclin D, and cyclin E were not affected. The decrease of cyclin A could be responsible for the lower Cdk2 activity observed at 15 h in W13-treated cells (Fig. 2B).

The two-dimensional pattern of Cdk4 on two-dimensional electrophoretic gels upon W13 treatment was also analyzed. As shown in Fig. 4, several spots (pI 5.3–5.9) were recognized by anti-Cdk4 antibodies. Although changes in the intensity of several spots were observed at 5 and 7 h after proliferative activation, no significant changes between W12- and W13-



**FIG. 4. Two-dimensional electrophoretic pattern of Cdk4.** Quiescent cells (Q), cells at 5 h after activation (5 h), and cells at 7 h (7 h) treated with W12 or W13 were lysed, and the proteins were separated by isoelectrofocusing (IEF). Then, they were electrophoresed on 10% SDS-polyacrylamide gels and transferred onto membranes as described under "Experimental Procedures." The blots were incubated with anti-Cdk4 antibodies.

treated cells were observed.

**CaM Inhibition Did Not Alter the Amount of p21 and p27**—Cells respond to different inhibitory stimuli by increasing the amount of the Cdk-inhibitory proteins, which bind to cyclin-Cdk4 and cyclin-Cdk2 complexes and inhibit its activity (23). Thus, we also analyzed here the possibility that W13 treatment induced an increase in the amount of p21 or p27. p21 was low in quiescent cells; it increased upon serum addition, and the levels remained constant between 5 and 15 h after activation. In contrast, the amount of p27 was high in quiescent cells and decreased upon serum addition. The treatment of the cells with W13 did not affect the levels of either inhibitor (Fig. 3).

**Effect of CaM Inhibition on the Formation of Cdk4 Complexes**—The possibility that CaM was involved in the formation of the cyclin D1-Cdk4 complexes was also analyzed here. Thus, cellular lysates immunoprecipitated with anti-cyclin D1 were electrophoresed and electroblotted, and the membranes were incubated with antibodies against cyclin D1, Cdk4, p27, and p21. As shown in Fig. 5, cyclin D1 present in NRK serum starved cells was already complexed with cdk4. In those complexes, high amounts of p27 were present, whereas almost no p21 was detected. At 7 h after activation, higher amounts of cyclin D1-cdk4 complexes were observed. Furthermore, the amount of p21 associated to cyclin D1 increased, whereas that of p27 decreased. The effect of CaM inhibition at 5 h after proliferative activation on the cyclin D1 complexes at 7 h was analyzed. As shown in Fig. 5, the amounts of Cdk4, p27, and p21 bound to cyclin D1 were not modified by the inactivation of CaM by W13-treatment or upon addition of the control drug W12. No change in the complexes was observed when immunoprecipitations were performed using anti-Cdk4 antibodies (data not shown).

**CaM Is Essential for the Nuclear Accumulation of Cdk4 and Cyclin D1**—Cdk4 and cyclin D1 are present in the nucleus during G<sub>1</sub> (47), which is in agreement with the nuclear location of the pRb family of proteins, the best known Cdk4 substrates. Immunocytochemical analysis indicated that the proportion of quiescent cells with nuclear staining for Cdk4 or for cyclin D1 was low (as also shown for cyclin D1 in Ref. 47). The percentage was higher at 5 h after proliferative activation, and at 7 h, almost all the nuclei were labeled in control cells (W12-treated cells and nontreated cells) (Fig. 6A). After W13 treatment, the percentage of cells at 7 h with Cdk4 and cyclin D1 nuclear

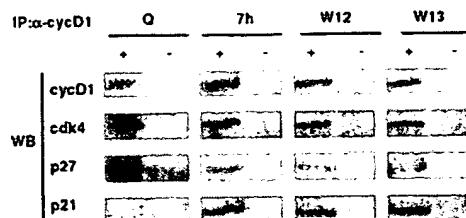


FIG. 5. Effect of anti-CaM drug addition on the association of Cdk4, p21, and p27 to cyclin D. Quiescent NRK cells (Q) were activated to proliferate, and at 5 h, W12 or W13 was added to the medium. Two hours later, the cells were harvested and processed for immunoprecipitation as indicated under "Experimental Procedures" with anti-cyclin D1 (cycD1) (+) or with normal rabbit serum (-) as a control. The immunoprecipitates were electrophoresed on 12% SDS-polyacrylamide gels and transferred onto membranes. The blots were incubated with antibodies against cyclin D1, Cdk4, p21, and p27. 7 h, cells lysed at 7 h without previous drug addition; W12 and W13, cells lysed at 7 h previously treated at 5 h with 15  $\mu$ g/ml of W12 or W13.

staining decreased dramatically to the values of quiescent cells (Figs. 6A and 7). The decrease of Cdk4 and cyclin D1 in the nucleus correlated with an increase in the cytoplasmic reactivity with both antibodies (Fig. 7), indicating that CaM was needed to maintain Cdk4 and cyclin D1 in the nucleus. Similar results were obtained using two different anti-Cdk4 antibodies and normal skin human fibroblasts (data not shown). The effect of CaM inhibition on the nuclear localization of Cdk4 and cyclin D1 was reversible because when W13 was removed 2 h after its addition and the cells were incubated for 2 h more in a medium without the drug, Cdk4 reentered the cell nucleus (90% of nuclei had a ratio of absorbance to area  $\geq 0.1$ ). In order to ensure the specificity of CaM inhibition, nuclear location of cdk4 was analyzed upon treatment of the cells with different anti-CaM drugs. As shown in Fig. 6B, the four anti-CaM drugs used, W13, W7, J8, and calmidazolium, induced in a dose-response manner a decrease in the nuclear staining of cdk4. Microinjection of the CaM-binding domain of CaMKII (CaMKII<sup>290-309</sup>) was also performed in order to inhibit CaM. Results showed a clear inhibition of DNA synthesis and Cdk4 nuclear accumulation after microinjection of CaMKII<sup>290-309</sup> peptide: 21  $\pm$  7% of CaMKII<sup>290-309</sup> peptide-injected cells were positive for BrdUrd *versus* 50  $\pm$  2% of control cells; and 42  $\pm$  10% of the CaMKII<sup>290-309</sup> peptide-injected cells had intense Cdk4-labeled nuclei (mean green  $\geq 100$ ) *versus* 85  $\pm$  3% of the control cells.

We also tried to analyze the amount of Cdk4 and cyclin D in purified nuclei, but unfortunately, with all the methods we have used (either with non-detergent-containing buffer or hypotonic buffers), more than 70% of both proteins leaked out from the nucleus and were found in the soluble fraction. Thus, it was impossible to analyze the effect of CaM inhibition on nuclear accumulation of cyclin D and Cdk4 by cell fractionation.

The effect of W13 treatment on the intracellular location of nucleolin and hnRNP A2 was also analyzed. Nucleolin contains a classical nuclear localization sequence (NLS); thus its transport to the nucleus is mediated by importin (48). HnRNP A2 has an M9 sequence that mediates its entrance to the nucleus via binding to transportin (49). Both proteins are known to shuttle in and out of the nucleus (48, 50). As shown in Fig. 7, CaM inhibition by W13 did not affect the nuclear location of either nucleolin or hnRNP A2.

A role of intact cytoskeleton in the nuclear transport of proteins that do not contain a canonical NLS, such as protein kinase C, has been shown (51). Thus, we analyzed the effect of actin filaments and microtubules disruption on nuclear localization of Cdk4. Cytochalasin D (1  $\mu$ g/ml) or nocodazole (1

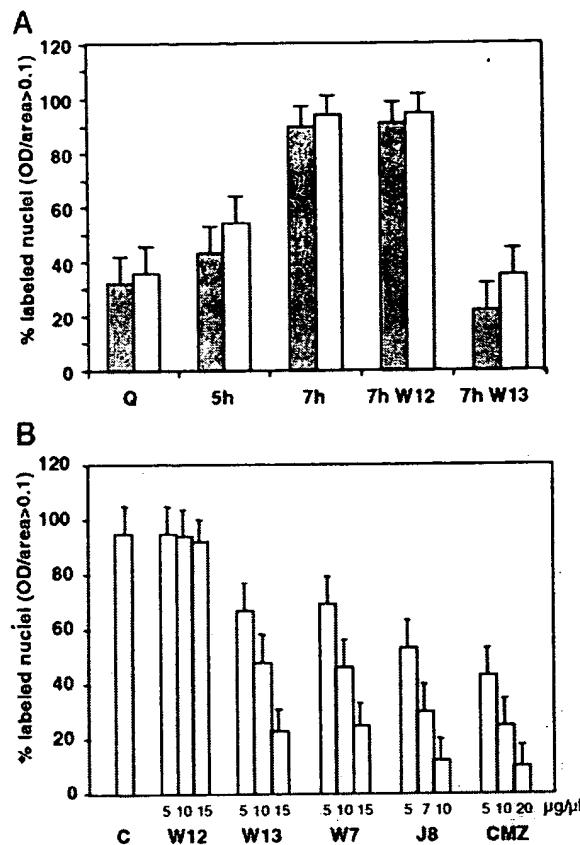


FIG. 6. Effect of anti-CaM drug treatment on the nuclear location of Cdk4 and cyclin D1. Quiescent NRK cells (Q) were subcultured on coverslips and at the same time activated to proliferate by serum addition. At 5 h after activation, the different anti-CaM drugs were added to the cultures, and at 7 h, cells were fixed and immunostained with anti-Cdk4 and anti-cyclin D1 as described under "Experimental Procedures" using peroxidase-conjugated secondary antibodies. The percentage of labeled nuclei was determined by immunocytochemistry and quantified by an Olympus image analysis. Nuclei were considered stained when the ratio of optical density to area was higher than 0.1. The results presented here are the mean of three different experiments. For each experiment, more than 200 nuclei were analyzed. A, filled bars indicate the percentage of cycD1-labeled nuclei, and empty bars indicate the percentage of Cdk4-labeled nuclei. W12 and W13 were added to a final concentration of 15  $\mu$ g/ml. B, % of Cdk4 labeled nuclei was measured after addition of W12, W13, W7, J8, and calmidazolium (CMZ) at the indicated concentrations or after no drug addition (C).

$\mu$ g/ml) treatment 5 h after proliferative activation did not have any effect on the nuclear staining of Cdk4 (data not shown).

Calcineurin inhibition by cyclosporin A (0.6–2.4  $\mu$ g/ml) or CaMKII inhibition by KN93 (5–10  $\mu$ M) treatment of the cells 5 h after proliferative activation did not have any effect on nuclear localization of Cdk4 or cyclin D1 (data not shown), indicating that these CaMBPs were not involved in cyclin D1-Cdk4 nuclear accumulation during G<sub>1</sub>.

**Cyclin D1 and Cdk4 Bind CaM**—In order to study whether the effect of CaM on the nuclear location of cyclin D1-Cdk4 was mediated by a CaM-binding protein associated to the complex, the binding of Cdk4 and cyclin D from cellular extracts to a CaM-affinity column was analyzed. Cell lysates containing EGTA were first applied to a DEAE-Sephacel column in order to eliminate endogenous CaM. Whereas Cdk4 appeared in the flow-through of the DEAE-Sephacel column, CaM was eluted at 600 mM salt (data not shown). After Ca<sup>2+</sup> addition, the DEAE-Sephacel flow-through was mixed with CaM-Sepharose resin. As shown in Fig. 8A, Cdk4 and cyclin D1 were able to bind to the CaM-Sepharose in the presence of Ca<sup>2+</sup> and eluted

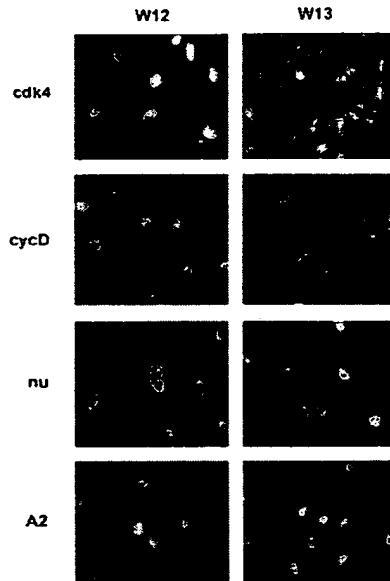


FIG. 7. Effect of W13 addition on the intracellular distribution of Cdk4 and cyclin D1. Quiescent NRK cells were subcultured on coverslips and at the same time activated to proliferate by serum addition. At 5 h after activation, 15  $\mu$ g/ml of W13 or W12 were added to the cultures; 2 h later, the cells were fixed and immunostained with anti-Cdk4 (Cdk4), anti-cyclin D1 (cycD), anti-nucleolin (nu), or anti-hnRNP A2 (A2) as described under "Experimental Procedures." Fluorescein-conjugated secondary antibodies were used in all cases.

specifically when EGTA was added. None of the proteins bound to a control Sepharose column. Bacterially expressed and purified Cdk4-GST and cyclin D1-GST were not found in the EGTA eluted fraction when applied to the CaM-Sepharose column under the same condition. To further prove that binding of Cdk4 and cyclin D1 to CaM was  $\text{Ca}^{2+}$ -dependent, pull-down experiments were performed. The flow-through of the DEAE-Sephadex column (in a buffer containing 5 mM EGTA) was divided and precipitated with CaM-Sepharose in the presence of either EGTA or free  $\text{Ca}^{2+}$ . Bound proteins were eluted with SDS-containing buffer. A replica of the  $\text{Ca}^{2+}$ -containing sample was also precipitated with control Sepharose. As shown in Fig. 8B, the binding of Cdk4 and cyclin D1 to the CaM-Sepharose was much higher in the presence of  $\text{Ca}^{2+}$  than of EGTA, and no binding was observed with the control Sepharose. In addition, CaM was found to immunoprecipitate with Cdk4 and with cyclin D1 (Fig. 8C). Hsp90, a CaM-binding protein that has recently been shown to associate with Cdk4 (52–54) was also detected in the EGTA-eluted fraction of the CaM-affinity column (Fig. 8A). Furthermore, in NRK cells, Hsp90 and Cdk4 also associate one to each other because Cdk4 was detected by Western blot in anti-Hsp90 immunoprecipitates from NRK lysates (Fig. 8C).

Because Hsp90 regulates the intracellular trafficking of several proteins (55), we analyzed whether inhibition of Hsp90 by the antibiotic geldanamycin had the same effect on intracellular location of cyclin D1-Cdk4 as CaM inhibition. Geldanamycin (2  $\mu$ M) addition to NRK cells 5 h after proliferative activation for a period of 2 h produced a clear reduction in nuclear Cdk4 and cyclin D1 (Fig. 9), whereas the total amount of Cdk4 analyzed by Western blot was not altered (data not shown).

#### DISCUSSION

Although it is well accepted that CaM has a role in the progression of  $G_1$  phase and in  $G_1$ -S transition, until now, the specific  $G_1$  steps regulated by CaM remain unclear. However, recent reports indicate that the inhibition of CaM during early  $G_1$  blocks the phosphorylation of pRb (15) and the expression of

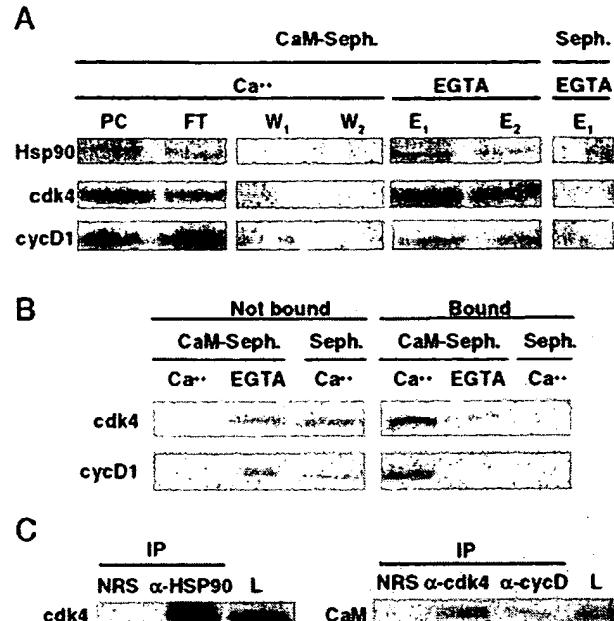


FIG. 8. Interaction of Cdk4 and cyclin D1 with CaM. A, binding of cyclin D1-Cdk4 to a calmodulin affinity column. Cellular extracts were loaded to a DEAE-Sephadex column as described under "Experimental Procedures," and the flow-through was applied to CaM-affinity Sepharose (CaM-Seph.) or to a control Sepharose column (Seph.) in the presence of free  $\text{Ca}^{2+}$ . After extensive washing in the presence of free  $\text{Ca}^{2+}$ , the bound proteins were specifically eluted with EGTA. Equivalent volumes of all the fractions were subjected to 10% SDS-polyacrylamide gels and analyzed by Western blotting using anti-Cdk4, anti-cyclin D1, and anti-Hsp90 antibodies. PC, precolumn; FT, flow-through;  $W_1$  and  $W_2$ , two last washes;  $E_1$  and  $E_2$ , EGTA, first and second specific elutions. B, flow-through of the DEAE column was incubated with CaM-Sepharose (CaM-Seph.) in the presence of EGTA or free  $\text{Ca}^{2+}$  or with control Sepharose (Seph.) in the presence of free  $\text{Ca}^{2+}$ . After extensive washing, bound proteins were eluted with Laemmli SDS-electrophoresis loading buffer. One-third of the unbound proteins and all of the bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the presence of Cdk4 and cycD1 was analyzed by Western blot. C, lysates of asynchronously proliferating NRK cells were immunoprecipitated as indicated under "Experimental Procedures" with anti-Hsp90, anti-Cdk4, anti-cyclin D1, or normal rabbit serum (NRS). The presence of Cdk4 in the Hsp90 immunoprecipitates was analyzed by Western blot. The presence of CaM in the Cdk4 and cycD1 immunoprecipitates was analyzed by Western blot. Cell lysates prior to immunoprecipitation were also loaded in the gels (L).

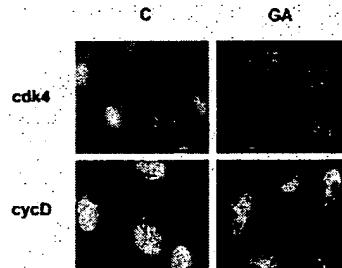


FIG. 9. Immunolocalization of Cdk4 and cyclin D1 after geldanamycin treatment. Quiescent NRK cells were subcultured on coverslips and at the same time activated to proliferate by serum addition. At 5 h after activation, 2  $\mu$ M geldanamycin (GA) dissolved in  $\text{Me}_2\text{SO}$  or 0.1%  $\text{Me}_2\text{SO}$  alone (C) was added; 2 h later, the cells were fixed and immunostained with anti-Cdk4 (cdk4) or anti-cyclin D1 (cycD) antibodies as described under "Experimental Procedures."

proteins involved in DNA replication (9), suggesting that the role of CaM in  $G_1$  progression is to regulate the activity of the kinases that phosphorylate pRb. In fact, we show here that CaM is essential for Cdk4 and Cdk2 activation. The role of CaM is not to regulate the expression of either Cdk4 or cyclin D1, the assembly of cyclin D1-Cdk4 complexes, or the binding of either

p21 or p27, but to maintain cyclin D1-Cdk4 complexes in the cell nucleus. Because these complexes are not in the nucleus after CaM inhibition, pRb cannot be phosphorylated; consequently, the expression of E2F-dependant genes is blocked, and the cell cycle stops before G<sub>1</sub>-S transition. The inhibition of CaM strongly decreased the levels of cyclin A and cdc2, proteins encoded by genes containing E2F-binding sites in their promoter regions, whereas in contrast, the levels of Cdk4, Cdk2, cyclin D1, p21, and p27, proteins the genes of which do not contain E2F-binding sites, were not affected by CaM inhibition. Although cyclin E also contains E2F-binding sites in its promoter region, its expression is not reduced by W13 treatment, indicating that a pRb phosphorylation-independent pathway of cyclin E expression exists in these cells.

Very little is known about the mechanisms of transport of Cdks and cyclins from the cytoplasm to the nucleus and *vice versa*. However, it is known that Cdk7 (the catalytic subunit of Cdk-activating kinase) is a nuclear protein that contains a classical NLS sequence that is responsible for its translocation from cytoplasm into the nucleus (56, 57). The intranuclear location of cyclin A depends of its association with Cdk2, although none of these proteins has NLS sequences (58). Cyclin D1 does not have a putative NLS, whereas Cdk4 has a potential classical NLS; thus, its import into the nucleus could be mediated by importin.

In quiescent cells, Cdk4 and cyclin D1 were located in the cytoplasm, but at 5–7 h after proliferative activation, both proteins were already in the nuclei. Inhibition of CaM at 5 h with W13 induced a release of Cdk4 and cyclin D from the nucleus to the cytosol in mid-G<sub>1</sub>, thus not allowing phosphorylation of pRb by cyclin D1-Cdk4. The general import of proteins into the nucleus was not affected upon anti-CaM drug addition, because the transport of nucleolin (mediated by importin) and hnRNP A2 (mediated by transportin) was not modified by the treatment. These results strongly suggest that CaM participates in the retention of cyclin D1-Cdk4 but does not regulate a general mechanism of nucleocytoplasmic transport.

A role for the CIP/KIP family of Cdk inhibitors on the cyclin D1-Cdk4 assembly and translocation to the nucleus has been proposed (59). Because CaM inhibition does not alter either Cdk4 and cyclin D1 assembly or the amount of p21 and p27 bound to those complexes, CaM action on nuclear accumulation of cyclin D1-Cdk4 must be downstream of the action of these inhibitors. Most recently, a residue of cyclin D1 (threonine 156) has been shown to be essential for the nuclear import of cyclin D1-Cdk4 but not essential for the assembly of the complex (60). Thus, in agreement with our results, assembly is not sufficient for nuclear translocation of cyclin D1-Cdk4. One possibility is that this cyclin D1 residue could be essential for the association of cyclin D1-Cdk4 with a CaMBP involved in the nuclear location of the complex.

It is worth mentioning that the inhibition of CaM also produced a strong decrease in Cdk4 activity. This decrease was not produced by a reduction in the amounts of Cdk4 and cyclin D1, nor was it due to either alterations in the association of Cdk4 to cyclin D1 or an increase in the levels of p27 and p21 bound to the complexes. An increase in the levels of inhibitors of the Ink4 family cannot be involved in the decrease of Cdk4 activity because they would interfere in the binding of Cdk4 to cyclin D1, and we did not find modifications of this association. Thus, the presence of cyclin D1-Cdk4 in the nucleus may be essential to the maintenance of the kinase activation. Because Cdk-activating kinase is located in the nucleus, when Cdk4 moves to the cytoplasm after CaM inhibition, this kinase cannot be phosphorylated by Cdk-activating kinase, and it then remains inactive. However, the two-dimensional pattern of Cdk4 observed

after W13-treatment was similar to that observed in W12-treated cells, suggesting, although not completely proving, that the phosphorylation state of Cdk4 is not altered when CaM is inhibited. It is also possible that the association of cyclin D1-Cdk4 with other proteins, or the phosphorylation of other proteins of the complex, could be essential for kinase activation in the nucleus or its inhibition in the cytoplasm. Mahony *et al.* (61) have recently shown that active cdk6 complexes are predominantly nuclear in T cells. Those complexes have a molecular mass determined by gel filtration chromatography of 170 kDa and contain cdk6, cyclin D, and other nonidentified proteins (61).

We propose two ways to explain how CaM inhibition induces the release of Cdk4 and cyclin D1 from the nucleus: 1) the kinase complex is moving continuously between the cytoplasm and the nucleus, and CaM is essential for its entrance into the nucleus; 2) the kinase complex is retained in the nucleus in a CaM-dependent manner. In relation to the first possibility, a GTP-independent and Ca<sup>2+</sup>/CaM-dependent nuclear protein import that would function at high cytoplasmic Ca<sup>2+</sup> concentrations has recently been described (62). According to those results, import of all NLS containing protein should be affected by CaM inhibition under these conditions, whereas in our model, nuclear accumulation of nucleolin was not modified. Related to the second possibility, it should be emphasized that CaM-binding proteins and CaM itself are present in the nucleus of proliferating cells (1). Thus, CaM could bind to cyclin D1-Cdk4 complexes, or alternatively, CaM could participate in a posttranslational modification (for example, phosphorylation or dephosphorylation) of cyclin D1-Cdk4 complexes, essential to retain these complexes in the nucleus. An example of such a mechanism has been recently reported for the nuclear location of the transcription factor NF-AT4. The presence of NF-AT4 in the nucleus depends on its association with calcineurin, a CaM-dependent phosphatase. CaM/calcineurin associates with NF-AT4 and keep it in a dephosphorylated state essential for its nuclear location (63).

We have shown that Cdk4 and cyclin D1 from a cell lysate are able to bind to CaM. Because purified Cdk4 and cyclin D1 do not bind CaM, they may form a complex with a CaMBP. We have analyzed the role of three different CaMBPs on the nuclear localization of cyclin D1-Cdk4: CaMKII, calcineurin, and Hsp90. Whereas CaMKII and calcineurin inhibition does not have any effect on nuclear localization of cyclin D1-Cdk4, Hsp90 inhibition by geldanamycin has the same effect as CaM inhibition. This, together with the facts that Hsp90 associates to Cdk4 also in NRK cells and that Hsp90 participates in the intracellular trafficking of several proteins (55), indicates that Hsp90 is a good candidate for being the CaMBP involved in the regulation of the nuclear accumulation of cyclin D1-Cdk4. It is also possible that Hsp90 is only associated to Cdk4, not to cyclin D1-Cdk4 complexes, and that Hsp90 is essential for nuclear accumulation of cdk4 in step previous to the one revealed using the anti-CaM drugs. Experiments to definitively determine the CaM-dependent steps and to identify the CaMBPs involved in nuclear location of cdk4 are under way in our laboratory.

Thus, we demonstrate here that CaM regulates the presence of cyclin D1-Cdk4 in the nucleus and consequently the phosphorylated state of pRb and G<sub>1</sub> progression. Inhibition of nuclear accumulation of cyclin B1 and thus cdc2 activity in response to the activation of DNA damage-induced G<sub>2</sub> checkpoint has also recently been shown (64). Our results reveal a novel mechanism for Cdk4 activity regulation that may operate in response to different extracellular signals or to the activation of cell cycle checkpoints in which Ca<sup>2+</sup> might be involved.

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# Differential Regulation of p27<sup>Kip1</sup> Expression by Mitogenic and Hypertrophic Factors: Involvement of Transcriptional and Posttranscriptional Mechanisms

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**Abstract.** Platelet-derived growth factor-BB (PDGF-BB) acts as a full mitogen for cultured aortic smooth muscle cells (SMC), promoting DNA synthesis and cell proliferation. In contrast, angiotensin II (Ang II) induces cellular hypertrophy as a result of increased protein synthesis, but is unable to drive cells into S phase. In an effort to understand the molecular basis for this differential growth response, we have examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery in rat aortic SMC. Both PDGF-BB and Ang II were found to stimulate the accumulation of G<sub>1</sub> cyclins with similar kinetics. In addition, little difference was observed in the expression level of their catalytic partners, Cdk4 and Cdk2. However, while both factors increased the enzymatic activity of Cdk4, only PDGF-BB stimulated Cdk2 activity in late G<sub>1</sub> phase. The lack of activation of Cdk2 in Ang II-treated cells was causally related to the failure of Ang II to stimulate phosphorylation of the enzyme on threo-

nine and to downregulate p27<sup>Kip1</sup> expression. By contrast, exposure to PDGF-BB resulted in a progressive and dramatic reduction in the level of p27<sup>Kip1</sup> protein. The time course of p27<sup>Kip1</sup> decline was correlated with a reduced rate of synthesis and an increased rate of degradation of the protein. Importantly, the repression of p27<sup>Kip1</sup> synthesis by PDGF-BB was associated with a marked attenuation of *Kip1* gene transcription and a corresponding decrease in *Kip1* mRNA accumulation. We also show that the failure of Ang II to promote S phase entry is not related to the autocrine production of transforming growth factor- $\beta$ 1 by aortic SMC. These results identify p27<sup>Kip1</sup> as an important regulator of the phenotypic response of vascular SMC to mitogenic and hypertrophic stimuli.

**Key words:** growth factors • cell cycle • CDK inhibitors • gene expression • smooth muscle cells

## Introduction

The proliferation of normal mammalian cells is controlled by an intricate network of biochemical pathways that ensure that each cell cycle event is performed correctly and in proper sequence (Murray and Hunt, 1993). Growth factor-induced signals are required for progression through the G<sub>1</sub> phase and must converge, in late G<sub>1</sub>, on the cell cycle engine to ensure the commitment of cells to enter S phase (Pardee, 1989). The regulation of G<sub>1</sub> progression and G<sub>1</sub>/S transition is governed, at least in part, by the con-

certed action of cyclin-dependent kinases (Cdks)<sup>1</sup> and their regulatory cyclin subunits (Draetta, 1994; Sherr, 1994; Grana and Reddy, 1995). When quiescent cells resume cycling in response to growth factors, D-type cyclins (D1, D2, and D3) progressively accumulate during G<sub>1</sub> phase and assemble with their catalytic partners, Cdk4 and Cdk6. The activity of Cdk4/Cdk6 is first detected in mid-G<sub>1</sub> and increases as cells approach the G<sub>1</sub>/S boundary. One major target of Cdk4/Cdk6 is the retinoblastoma pro-

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<sup>1</sup>Abbreviations used in this paper: Ang II, angiotensin II; CAK, Cdk-activating kinase; Cdk, cyclin-dependent kinase; DRB, 5,6-Dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; MAP, mitogen-activated protein; PDGF-BB, platelet-derived growth factor-BB; pRb, retinoblastoma protein; SMC, smooth muscle cells; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNA, TGF- $\beta$ 1 neutralizing antibody.

tein (pRb), which upon phosphorylation dissociates from bound transcription factors, such as E2F, enabling them to activate genes required for DNA replication (Weinberg, 1995). Cyclin E is expressed at maximum level in late G<sub>1</sub> and associates with Cdk2. Biochemical and genetic data indicate that cyclin E-Cdk2 activity is essential for entry into S phase (van den Heuvel and Harlow, 1993; Knoblich et al., 1994; Ohtsubo et al., 1995; Krude et al., 1997).

The activity of Cdk5 is regulated by a combination of mechanisms. These include the synthesis of the cyclin and Cdk, the assembly of these proteins into complexes, the phosphorylation of a conserved threonine residue by Cdk-activating kinase (CAK), and the interaction with Cdk inhibitory proteins (Morgan, 1995). Cdk inhibitors fall into two gene families (Sherr and Roberts, 1995). The Ink4 family of proteins, which includes p16<sup>Ink4A</sup>, p15<sup>Ink4B</sup>, p18<sup>Ink4C</sup>, and p19<sup>Ink4D</sup>, specifically interacts with Cdk4 and Cdk6 to prevent cyclin D-Cdk assembly or enters into stable ternary complexes with cyclin D-Cdk, resulting in complexes that are catalytically inactive (Serrano et al., 1993; Guan et al., 1994; Hannon and Beach, 1994; Chan et al., 1995; Hirai et al., 1995). The second family of inhibitors includes p21<sup>Cip1/Waf1</sup> (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994), p27<sup>Kip1</sup> (Polyak et al., 1994a; Toyoshima and Hunter, 1994), and p57<sup>Kip2</sup> (Lee et al., 1995; Matsuoka et al., 1995), which are all structurally unrelated to the Ink4 proteins. The Cip/kip family binds to and inhibits a broader range of Cdk5 than the Ink4 family and displays a preference for fully assembled cyclin-Cdk complexes. They inhibit the kinase activity of G<sub>1</sub> Cdk5 by stoichiometric binding to the cyclin-Cdk complex or by physically blocking the phosphorylation of the Cdk subunit by CAK (Sherr and Roberts, 1995). Among them, p27<sup>Kip1</sup> was first identified in transforming growth factor  $\beta$  (TGF- $\beta$ )-treated cells (Polyak et al., 1994b; Slingerland et al., 1994). The expression of p27<sup>Kip1</sup> is increased in serum-starved or density-arrested cells (Firpo et al., 1994; Kato et al., 1994; Nourse et al., 1994) and in cells exposed to antiproliferative signals like TGF- $\beta$ , rapamycin (Nourse et al., 1994), and cAMP (Kato et al., 1994; L'Allemand et al., 1997). In contrast, the level of p27<sup>Kip1</sup> declines in response to mitogenic factor stimulation (Kato et al., 1994; Nourse et al., 1994; Coats et al., 1996; Winston et al., 1996; this study). Thus, in addition to D-type cyclins, p27<sup>Kip1</sup> may play an essential role in connecting mitogenic signaling pathways to cell cycle activation. Ectopic expression of p27<sup>Kip1</sup> causes cell cycle arrest in G<sub>1</sub> phase (Polyak et al., 1994a; Toyoshima and Hunter, 1994) and, conversely, antisense inhibition of p27<sup>Kip1</sup> expression suppresses quiescence in fibroblasts (Coats et al., 1996; Rivard et al., 1996).

In cultured arterial smooth muscle cells (SMC), the peptide growth factor platelet-derived growth factor (PDGF)-BB acts as a full mitogen, promoting DNA synthesis and cell division (Raines et al., 1990; Grainger et al., 1994). The mitogenic action of PDGF-BB is initiated by its interaction with two structurally related tyrosine kinase receptors that dimerize upon ligand binding, leading to activation of the intrinsic kinase domain and intermolecular autophosphorylation (Claesson-Welsh, 1994). The phosphorylated tyrosine residues serve as docking sites for multiple SH2-containing signaling molecules that include

Src, phosphoinositide 3-kinase (PI3-kinase), phospholipase C- $\gamma$  (PLC- $\gamma$ ), SHP-2, Grb2, Shc, and Nck. Recruitment and activation of these effector proteins catalyze the formation of second messengers and propagate the signal to downstream serine/threonine kinases, such as protein kinase C, mitogen-activated protein (MAP) kinases, and p70 S6 kinase, ultimately resulting in increased gene expression and DNA synthesis (Claesson-Welsh, 1994; Heldin, 1997).

In contrast to PDGF-BB, many investigators, including ourselves, have shown that the peptide angiotensin II (Ang II) induces cellular hypertrophy in cultured aortic SMC as a result of increased protein synthesis, but is unable to drive cells into S phase (Geisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Giasson and Meloche, 1995). On the other hand, Ang II was reported to exert weak mitogenic effects on SMC of resistance arteries (Dubey et al., 1992) and on aortic SMC isolated from spontaneously hypertensive rats (Bunkenburg et al., 1992; Itazaki et al., 1995). In vivo, a number of studies have shown that infusion of Ang II stimulates SMC DNA synthesis and proliferation in normal and injured rat arteries (Daemen et al., 1991; van Kleef et al., 1992; de-Blois et al., 1996; Su et al., 1998). However, results of in vivo studies are difficult to interpret since the effect of Ang II may be indirect or Ang II may simply act as a mitogen. It has been postulated that Ang II may be a bifunctional growth factor that activates both proliferative and antiproliferative (TGF- $\beta$ 1) signals in vascular SMC (Gibbons et al., 1992; Koibuchi et al., 1993). According to this model, the autocrine production of TGF- $\beta$ 1 would determine whether vascular SMC grow by hypertrophy or hyperplasia in response to Ang II.

In cultured aortic SMC, the hypertrophic action of Ang II is initiated by its interaction with the G protein-coupled AT<sub>1</sub> receptor, which stimulates the activity of PLC- $\beta$  to generate the second messengers inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) and diacylglycerol, and inhibits the activity of adenylyl cyclase (Catt et al., 1993; Timmermans et al., 1993). These early signaling events subsequently lead to the activation of multiple serine/threonine kinases, which include the MAP kinases ERK1/ERK2 (Duff et al., 1992; Tsuda et al., 1992; Servant et al., 1996) and p70 S6 kinase (Giasson and Meloche, 1995). Ang II also induces tyrosine phosphorylation of multiple proteins in aortic SMC (Molloy et al., 1993; Leduc et al., 1995) and stimulates the activity of cytosolic tyrosine kinases, such as p125<sup>FAK</sup> (Polte et al., 1994; Giasson et al., 1997), Pyk2 (Giasson et al., 1997), Src (Ishida et al., 1995), and the Janus kinases Jak2 and Tyk2 (Marrero et al., 1995; Giasson et al., 1997). Despite the fact that Ang II and PDGF-BB activate similar signal transduction pathways, only the latter is able to induce proliferation of aortic SMC.

In an effort to understand the molecular basis for this differential response, we have examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery. We show that while both factors are able to stimulate the activity of Cdk4, only PDGF-BB increases the enzymatic activity of Cdk2 in late G<sub>1</sub> phase. The lack of activation of Cdk2 in Ang II-treated cells is associated with the failure of Ang II to downregulate p27<sup>Kip1</sup> expression. We also show that p27<sup>Kip1</sup> abundance is regulated by

multiple transcriptional and posttranscriptional mechanisms in vascular SMC.

## Materials and Methods

### Cell Culture

Rat aortic SMC were cultured to 80% confluence and synchronized in the quiescent state as described previously (Giasson and Meloche, 1995). The cells were stimulated with 100 nM Ang II (Hukabel Scientific) or 50 ng/ml PDGF-BB (Oncogene Science) for the indicated times at 37°C. Mv1Lu mink lung epithelial cells (obtained from Dr. Maureen O'Connor, Biotechnology Research Institute of Montreal, Canada) were grown in MEM containing 10% FBS.

### Protein Synthesis, DNA Synthesis, and Cell Number Measurements

For protein synthesis measurements, quiescent aortic SMC in 6-well plates were stimulated with Ang II or PDGF-BB for 72 h in serum-free medium containing 0.5  $\mu$ Ci/ml [ $^3$ H]leucine. For DNA synthesis measurements, quiescent aortic SMC in 35-mm petri dishes were stimulated for the indicated times with Ang II or PDGF-BB and pulse-labeled with 2  $\mu$ Ci/ml [ $^3$ H]thymidine for the last 2–4 h. After the stimulation, the medium was aspirated and the cells were incubated for a minimum of 30 min in cold 5% TCA. The wells were then washed once with TCA and three times with tap water. The radioactivity incorporated into TCA-precipitable material was measured by liquid scintillation counting after solubilization in 0.1 M NaOH. For determination of cell number, quiescent aortic SMC in 6-well plates were stimulated with Ang II or PDGF-BB for 72 h and then were trypsinized and counted using a hemacytometer.

### Immunoblot Analysis

Cells were washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 10 $^{-4}$  M phenylmethylsulfonyl fluoride, 10 $^{-6}$  M leupeptin, 10 $^{-6}$  M pepstatin A, 1% Triton X-100) for 30 min at 4°C. Lysates were clarified by centrifugation at 13,000  $\times$  g for 10 min and equal amounts of lysate proteins (30–85  $\mu$ g) were subjected to electrophoresis on 12 or 15% acrylamide gels. Proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Nycomed Amersham, Inc.) in 25 mM Tris, 192 mM glycine, and fixed for 10 min in methanol/acetic acid/glycerol (40:7:3). The membranes were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h at 37°C before incubation for 1 h at 25°C with 2  $\mu$ g/ml of mAb to cyclin D1 (DCS-6), cyclin D2 (DCS-3.1), or cyclin D3 (DCS-22; NeoMarkers), or 1  $\mu$ g/ml of polyclonal antibody to cyclin E (SC-481), Cdk2 (SC-163), Cdk4 (SC-260), or p27<sup>Kip1</sup> (SC-528; Santa Cruz Biotechnology) in blocking solution. After washing four times in TBS, 0.1% Tween 20, the membranes were incubated for 1 h with HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:10,000) in blocking solution. Immunoreactive bands were visualized by enhanced chemiluminescence (Nycomed Amersham, Inc.).

For coprecipitation studies, total lysate proteins (200–500  $\mu$ g) were incubated for 3 h at 4°C with anticyclin E antibody and the immune complexes were collected with protein A-Sepharose beads (Pharmacia Biotech). The beads were washed five times with Triton X-100 lysis buffer, resuspended in denaturing sample buffer, and the eluted proteins were analyzed by immunoblotting.

### Protein Kinase Assays

The phosphotransferase activity of Cdk2 was measured by immune complex kinase assay using histone H1 as substrate as described previously (Meloche, 1995). In brief, lysate proteins (200  $\mu$ g) were subjected to immunoprecipitation with 1  $\mu$ g of anti-Cdk2 antibody preadsorbed to protein A-Sepharose beads for 2 h at 4°C. The immune complexes were washed three times with Triton X-100 lysis buffer and once with kinase assay buffer (20 mM Hepes, pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol). Histone H1 kinase activity was assayed by resuspending the beads in a total volume of 40  $\mu$ l of kinase assay buffer containing 0.25 mg/ml histone H1 (Boehringer Mannheim Corp.), 100  $\mu$ M ATP, and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP. The reactions were initiated by the addition of ATP, incubated at 30°C for

5 min, and stopped by addition of 2 $\times$  denaturing sample buffer. The samples were analyzed by SDS-gel electrophoresis and the bands corresponding to histone H1 were excised and counted.

For inhibition experiments, extracts of PDGF-BB-stimulated cells containing active Cdk2 were mixed with boiled (5 min at 100°C) extracts of Ang II-stimulated cells (1:1 ratio; 200  $\mu$ g protein of each lysate) for 1.5 h at 4°C before immunoprecipitation of Cdk2 and kinase assay. Immunodepletion of p27<sup>Kip1</sup> was performed by incubating 200  $\mu$ g of Ang II-treated cell extract with 5  $\mu$ g of anti-p27<sup>Kip1</sup> antibody for 2 h at 4°C. The resulting supernatant was then used for the inhibition experiment. Specificity of p27<sup>Kip1</sup> immunodepletion was assessed by preincubating the anti-p27<sup>Kip1</sup> antibody with excess immunogenic peptide (50  $\mu$ g of SC-528P; Santa-Cruz Biotechnology) for 2 h at 4°C before incubation with Ang II-treated cell extract.

Cdk4 enzymatic assays were performed as described (Matsushime et al., 1994) with some modifications. After stimulation, the cells were washed twice with ice-cold PBS and lysed in Tween 20 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM EGTA, 20 mM  $\beta$ -glycerophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 $^{-4}$  M phenylmethylsulfonyl fluoride, 10 $^{-6}$  M leupeptin, 10 $^{-6}$  M pepstatin A, and 0.1% Tween 20). The cells were scraped from the plates and sonicated at 4°C (once for 10 s). Cellular lysates were clarified by centrifugation and 150  $\mu$ g of lysate proteins were precleared for 1 h with 5  $\mu$ l of normal rabbit serum and then incubated for 3 h at 4°C with 1  $\mu$ g of anti-Cdk4 antibody preadsorbed to protein A-Sepharose beads. The immune complexes were washed twice with Tween 20 lysis buffer and twice with kinase assay buffer (50 mM Hepes, pH 7.4, 10 mM MgCl<sub>2</sub>, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM  $\beta$ -glycerophosphate, 1 mM sodium fluoride, and 0.1 mM sodium orthovanadate). pRb kinase activity was assayed by resuspending the beads in a total volume of 40  $\mu$ l of kinase assay buffer containing 1  $\mu$ g glutathione S-transferase (GST)-pRb protein (amino acids 792–928), 0.2 mg/ml BSA, 20  $\mu$ M ATP, and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP. The reactions were incubated at 30°C for 30 min and stopped by addition of 2 $\times$  denaturing sample buffer. The samples were resolved by SDS-gel electrophoresis and the radioactivity incorporated into GST-pRb was counted.

The enzymatic activity of CAK was measured essentially as described (Musgrove et al., 1998). Cellular extracts (400  $\mu$ g protein) prepared in CAK lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 10 mM  $\beta$ -glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 $^{-4}$  M phenylmethylsulfonyl fluoride, 10 $^{-6}$  M leupeptin, 10 $^{-6}$  M pepstatin A, and 0.1% Tween 20) were precleared as described above and then incubated for 3.5 h at 4°C with 5  $\mu$ g of anti-Cdk7 antibody (06-377; Upstate Biotechnology) preadsorbed to protein A-Sepharose beads. The immune complexes were washed twice with lysis buffer and twice with kinase assay buffer (50 mM Hepes, pH 7.5, 30 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol). CAK activity was assayed by resuspending the beads in 40  $\mu$ l of kinase assay buffer containing 5  $\mu$ g of GST-Cdk2K33M, 90  $\mu$ M ATP, and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP. The reactions were incubated for 20 min at 30°C and stopped by addition of 2 $\times$  denaturing sample buffer. The samples were analyzed by SDS-gel electrophoresis and the bands corresponding to GST-Cdk2K33M were excised and counted. No CAK kinase activity was detected in samples subjected to immunoprecipitation with beads alone.

The recombinant GST fusion proteins of pRb and Cdk2K33M were expressed in *Escherichia coli* by transformation with plasmids pGEX-Rb and pGEX-Cdk2K33M (obtained from Drs. Jacques Pouysségur, Centre de Biologie-CNRS, Nice, France, and Tomi P. Mäkelä, University of Helsinki, Helsinki, Finland, respectively) and purified as described (Matsushime et al., 1994).

### Phosphorous 32 Labeling and Immunoprecipitation

Quiescent aortic SMC in 100-mm petri dishes were stimulated for 10 or 20 h with Ang II or PDGF-BB and labeled for the last 5 h in bicarbonate- and phosphate-free Hepes-buffered MEM containing 0.5 mCi/ml [ $^{32}$ P]phosphoric acid. The cells were then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer. After clarification, the lysates were precleared for 1 h with 5  $\mu$ l of normal rabbit serum and Cdk2 was immunoprecipitated as described above. Immune complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 10% acrylamide gels. The proteins were then electrophoretically transferred to PVDF membranes (Millipore) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography.

## Phosphoamino Acid Analysis

The labeled band corresponding to Cdk2 was excised from the PVDF membrane and subjected to partial acid hydrolysis in 5.7 M HCl for 1 h at 110°C (Kamps, 1991). The resulting phosphoamino acids, along with unlabeled phosphoamino acid standards (0.2 mg/ml), were separated by one-dimensional thin layer electrophoresis using an optimized pH 2.5 buffer (Jelinek and Weber, 1993). The standards were visualized by ninhydrin staining and the labeled amino acids by autoradiography.

## Biosynthetic Labeling Experiments

To examine the turnover of p27<sup>Kip1</sup> protein, quiescent aortic SMC in 100-mm petri dishes were pulse-labeled for 1 h with 166  $\mu$ ci/ml of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine and then chased for the indicated times in serum-free medium containing excess methionine and cysteine and either Ang II or PDGF-BB. The cells were then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer. Lysates (500  $\mu$ g proteins) were pre-cleared for 1 h with 5  $\mu$ l of normal rabbit serum and the resulting supernatants were incubated with protein A-Sepharose beads preadsorbed with 2  $\mu$ g of anti-p27<sup>Kip1</sup> for 4 h at 4°C. Immune complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The p27<sup>Kip1</sup> protein was detected by fluorography and quantified using a PhosphorImager apparatus.

For labeling newly synthesized proteins, cells were stimulated for the indicated times, rinsed with methionine- and cysteine-free medium, and incubated with 250  $\mu$ ci/ml of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. Labeling was allowed to proceed for the last 20 min. Cell lysis and immunoprecipitation of p27<sup>Kip1</sup> were conducted as described above.

## Northern Blot Analysis

Total RNA was extracted by a modified version of the guanidinium thiocyanate procedure as described (Chomczynski and Sacchi, 1987; Chomczynski, 1993). Equal amounts of total RNA (15–25  $\mu$ g) were denatured and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA was transferred to Hybond-N membranes (Nycomed Amersham, Inc.), fixed, and hybridized with <sup>32</sup>P-labeled probes. Hybridization was carried out in hybridization medium (5  $\times$  SSC [ $1 \times$  SSC = 150 mM NaCl, 15 mM sodium citrate], 0.1% SDS, 5  $\times$  Denhardt's solution [ $1 \times$  Denhardt's = 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, and 0.02% BSA], 50% formamide, and 100  $\mu$ g/ml herring sperm DNA) containing the labeled probe (1–2  $\times$  10<sup>6</sup> cpm/ml) for 16 h at 42°C. The membranes were washed twice at 25°C for 15 min in 2  $\times$  SSC, 0.1% SDS, and twice at 60°C for 30 min in 0.5  $\times$  SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus. The results were normalized to 18S ribosomal RNA.

The probes used were: 1.5-kb EcoRI fragment of human p27<sup>Kip1</sup> cDNA (provided by Dr. Joan Massagué, Memorial Sloan-Kettering Cancer Center, NY) and a DNA oligonucleotide derived from the rat 18S ribosomal RNA sequence.

## Nuclear Run-On Transcription Assays

Nuclei were prepared as described by Greenberg and Bender (1997). Vascular SMC were washed twice with ice-cold PBS and scraped from plates in PBS, 1 mM EDTA. Cell pellets were collected by centrifugation and resuspended in cold lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1% NP-40). The cells were then disrupted in a Dounce homogenizer and the nuclei were sedimented at 500 g for 5 min. The nuclei were resuspended in 50 mM Tris-HCl, pH 8.3, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 40% glycerol and frozen in liquid nitrogen. For run-on transcription reactions, thawed nuclei ( $8 \times 10^7$ ) were resuspended in 400  $\mu$ l of reaction buffer containing 5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl<sub>2</sub>, 150 mM KCl, 5 mM dithiothreitol, 20 U/ml RNA guard (Pharmacia Biotech), 1 mM each of ATP, GTP, and CTP, and 600  $\mu$ ci of  $\alpha^{32}$ P]UTP (Nycomed Amersham, Inc.: 800 Ci/mmol) and incubated at 37°C for 30 min. Transcription was stopped by the addition of 40  $\mu$ g DNase I in 1 ml of HSB buffer and incubated for 5 min at 30°C. Then, 10  $\mu$ l of 20  $\mu$ g/ml proteinase K in 0.5 M Tris-HCl, pH 7.4, 125 mM EDTA, and 5% SDS was added to the reaction mixture, followed by incubation for 30 min at 42°C. The <sup>32</sup>P-labeled RNA was extracted with phenol/chloroform and unincorporated nucleotides were removed by chromatography through a Sephadex G-50 (Pharmacia Biotech) column. Each plasmid DNA gene insert was denatured and immobilized on nitrocellulose membranes using a dot-blot

apparatus. The membranes were hybridized with <sup>32</sup>P-labeled RNA in 5  $\times$  SSC, 5  $\times$  Denhardt's, 50% formamide, 4 mM EDTA, 0.5 mg/ml salmon sperm DNA, and 0.25 mg/ml yeast tRNA at 55°C for 24 h. The membranes were washed extensively at 60°C in 0.5  $\times$  SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus.

## $^3$ H]Uridine Pulse-Chase Experiments

Quiescent aortic SMC were pretreated for 2 h with 20 mM glucosamine (Sigma Chemical Co.) to deplete the UTP pool, washed, and pulse-labeled with 100  $\mu$ ci/ml [ $^3$ H]uridine (26 Ci/mmol; Nycomed Amersham, Inc.) for 12 h. The unincorporated [ $^3$ H]uridine-containing medium was removed and the cells were incubated for an additional 2 h in serum-free medium containing 20 mM glucosamine, 5 mM uridine, and 5 mM cytidine. The chase was then continued for 4 h in the same medium containing Ang II or PDGF-BB. At various intervals, the cells were washed with PBS and total RNA was isolated as described above. Equivalent amounts of  $^3$ H-labeled RNA were hybridized to 5  $\mu$ g of linearized plasmid DNA immobilized onto a nitrocellulose membrane. Hybridization was performed at 45°C for 4 d, as described in the previous section. The membranes were washed extensively at 55°C in 0.5  $\times$  SSC, 0.1% SDS. After drying, the radioactivity of each spot was determined by liquid scintillation counting.

## TGF- $\beta$ 1 Bioassay

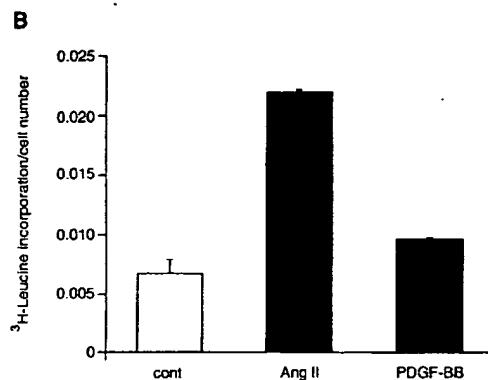
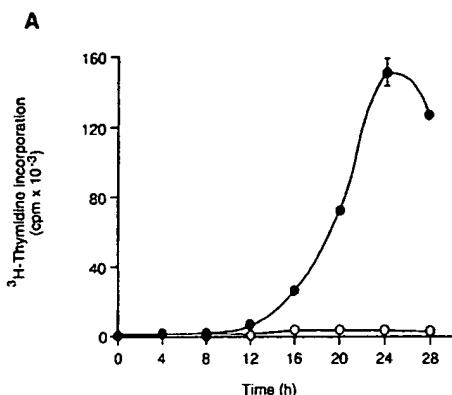
TGF- $\beta$ 1 bioassay was conducted essentially as described previously (Gibbons et al., 1992). Recombinant TGF- $\beta$ 1 and TGF- $\beta$ 1 neutralizing antibody (TNA) was a generous gift from Dr. Maureen O'Connor. In brief, Mv1Lu cells were plated at a density of  $5 \times 10^5$  cells per well in 24-well plates. After 6 h of serum exposure, the cells were washed with serum-free medium, then incubated with conditioned medium or TGF- $\beta$ 1 in serum-free medium for 24 h. The rate of DNA synthesis was measured by pulse-labeling cells with 2  $\mu$ ci/ml [ $^3$ H]thymidine during the last 6 h of incubation. For each experiment, a standard curve was constructed with increasing concentrations of recombinant TGF- $\beta$ 1. Ang II conditioned medium was obtained from aortic SMC stimulated for 24 h with Ang II and was added to Mv1Lu cells at two dilutions (1:5 and 1:10).

TNA was purified by protein A-agarose chromatography. The antibody was used at a concentration of 10–15  $\mu$ g/ml, which completely blocks the growth inhibitory effect of TGF- $\beta$ 1 in Mv1Lu cells.

## Results

### PDGF-BB, but Not Ang II, Induces DNA Synthesis in Aortic SMC

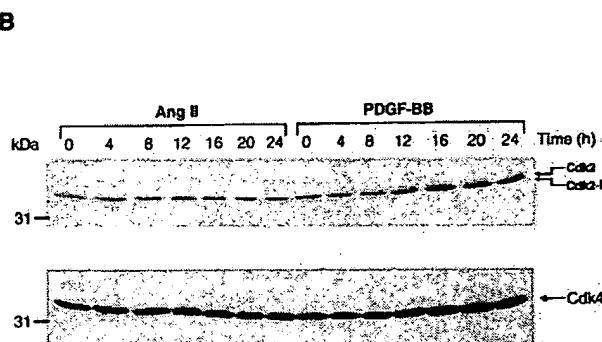
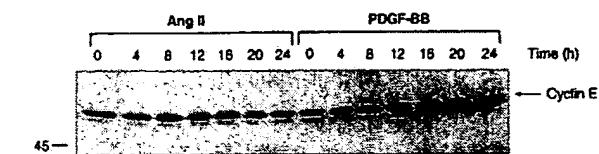
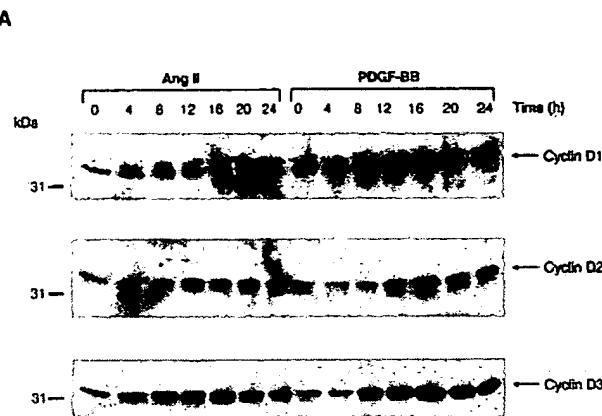
We compared the ability of the vascular growth factors PDGF-BB and Ang II to stimulate the rate of DNA synthesis in quiescent rat aortic SMC. As previously reported (Geisterer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Giasson and Meloche, 1995), treatment of aortic SMC with Ang II had no significant effect on DNA synthesis, as measured by [ $^3$ H]thymidine incorporation (Fig. 1 A). In contrast, addition of PDGF-BB strongly increased the rate of DNA synthesis, which reached a peak (180-fold over basal level) at 24 h after stimulation. PDGF-BB also induced cellular division as reflected by an increase in SMC number and by the small ratio between [ $^3$ H]leucine incorporation and cell number (Fig. 1 B). Ang II did not promote cell division, but caused cellular hypertrophy by increasing the rate of protein synthesis per cell over a period of 72 h (Fig. 1 B). This finding suggests that cell cycle progression is not delayed in Ang II-stimulated cells, but rather, that cells are arrested in G<sub>1</sub> phase. Thus, PDGF-BB is a strong mitogenic factor for rat aortic SMC, promoting DNA synthesis and cellular division, whereas Ang II acts as a hypertrophic factor.



**Figure 1.** Comparative effects of Ang II and PDGF-BB on the growth of aortic SMC. A, DNA synthesis. Quiescent rat aortic SMC were stimulated for different periods of time with 100 nM Ang II (○) or 50 ng/ml PDGF-BB (●) and the rate of DNA synthesis was measured by [<sup>3</sup>H]thymidine incorporation. Each value represents the mean  $\pm$  SEM of triplicate determinations. B, Cell protein content. Quiescent aortic SMC were stimulated with Ang II or PDGF-BB for 72 h. The rate of protein synthesis was measured by [<sup>3</sup>H]leucine incorporation and the number of cells counted on parallel plates. The results are expressed as the relative ratio between [<sup>3</sup>H]leucine incorporation and cell number. Each value represents the mean  $\pm$  SEM of triplicate determinations. Cell numbers for this experiment were: control (cont), 374,167  $\pm$  53,428 cells; Ang II, 312,000  $\pm$  28,369 cells; PDGF-BB, 917,500  $\pm$  42,120 cells. The data are representative of at least three different experiments with similar results.

### Ang II Fails to Stimulate the Activity of Cdk2 in Aortic SMC

In an effort to understand the molecular basis for this differential response of aortic SMC to vascular growth factors, we examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery. We first analyzed the regulated expression of G<sub>1</sub> cyclins. Fig. 2 A shows that both PDGF-BB and Ang II stimulated the accumulation of D-type cyclins with similar kinetics. The expression of cyclin D1, D2, and D3 started to increase at 4 h poststimulation and reached a maximal level by 12–16 h. Cyclin E expression was already detectable in quiescent cells. Treatment with PDGF-BB caused a small but significant increase in cyclin E expression, which was delayed compared with D-type cyclins (Fig. 2 A). Ang II had little effect on cyclin E expression. It should also be noted that

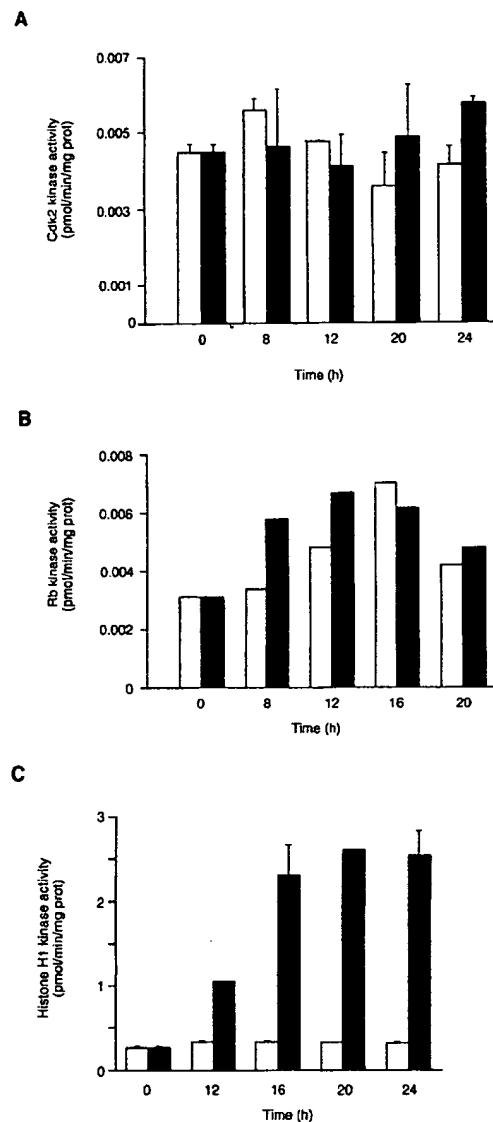


**Figure 2.** Effects of Ang II and PDGF-BB on the accumulation of G<sub>1</sub> cyclins and the expression level of Cdks in aortic SMC. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis on 12% acrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with antibodies specific to the different cyclins and Cdks. The proteins were visualized by chemiluminescence detection. Similar results were obtained in five different experiments.

PDGF-BB promoted higher levels of cyclin D1 accumulation than Ang II in aortic SMC. This is in agreement with previous observations showing that the extent of cyclin D1 accumulation is correlated with the mitogenic potential of growth factors and their ability to induce sustained ERK1/ERK2 activation (Lavoie et al., 1996; and data not shown). Little difference was observed in the expression level of the catalytic subunits Cdk4 and Cdk2, which were present in all extracts, including those prepared from quiescent cells (Fig. 2 B). However, we noted that treatment with PDGF-BB results in the late appearance of a faster migrating species of Cdk2, which is indicative of phosphorylation of the enzyme on threonine 160 (Gu et al., 1992). Only the slower migrating form of Cdk2 was observed in Ang II-stimulated cells.

We next measured the enzymatic activity of CAK, Cdk4, and Cdk2 after treatment of aortic SMC with the two vascular growth factors. CAK, Cdk4, and Cdk2 were selectively immunoprecipitated from cell lysates and their activity assayed in vitro using GST-Cdk2, GST-pRb, and histone H1 as substrates, respectively. As observed in other cellular models (Sclafani, 1996), CAK enzymatic activity was the same in quiescent and growth factor-treated aortic SMC (Fig. 3 A). Both Ang II and PDGF-BB increased

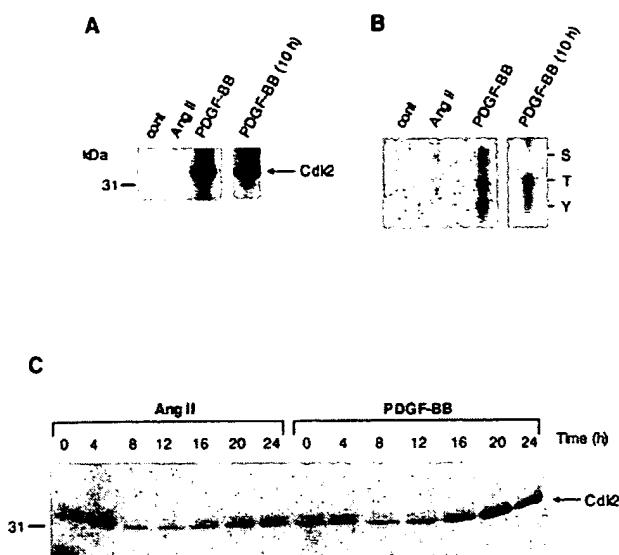
the Rb kinase activity of Cdk4, which became detectable at eight hours and remained elevated up to the end of G<sub>1</sub> phase (Fig. 3 B). Notably, Ang II treatment of aortic SMC induced a delayed Rb kinase activity as compared with PDGF-BB treatment. These results most likely reflect the quantitative differences in the ability of Ang II and PDGF-BB to regulate the expression of cyclin D1 (Fig. 2 A). Both factors equally stimulated Cdk4 activity after 16 h of exposure. As expected, stimulation of aortic SMC with the mitogenic factor PDGF-BB strongly increased Cdk2-associated histone H1 kinase activity, which was first detected at 12 h and reached a maximum in S phase (Fig. 3 C). In contrast, treatment with Ang II failed to induce any detectable Cdk2 activity over the same period of time. Thus, we carried out a series of experiments to explain the inability of Ang II to activate Cdk2. Since CAK-mediated phosphorylation of threonine 160 on Cdk2 is required for kinase activation (Morgan, 1995), we first analyzed the phosphorylation state of Cdk2 after immunoprecipitation from <sup>32</sup>P-labeled cells stimulated with Ang II or PDGF-BB. Fig. 4 A shows that the lack of activation of Cdk2 in Ang II-treated cells was associated with the failure of Ang II to stimulate phosphorylation of the enzyme on threonine. On the other hand, addition of PDGF-BB resulted in the phosphorylation of Cdk2 on threonine, tyrosine, and serine residues after 20 h (Fig. 4 B). Indeed, it has been reported in HeLa cells that most of the phosphorylation of Cdk2 on tyrosine (tyrosine 15) occurs on Cdk2 molecules that are also phosphorylated on threonine 160 (Gu et al., 1992). The absence of CAK-mediated threonine phosphorylation of Cdk2 in Ang II-treated cells was not attributable to the inability of Cdk2 to form complexes with cyclin E. Immunoblot analysis showed that cyclin E immunoprecipitates from quiescent aortic SMC already contained a significant amount of Cdk2 and that treatment with PDGF-BB caused a further increase in complex formation that became apparent only after 16 h of stimulation (Fig. 4 C). In addition, detailed kinetic analysis of Cdk2 phosphorylation and activity revealed that 10–12 h of PDGF-BB stimulation (thus before induction of cyclin E expression and increased cyclin E–Cdk2 complex formation) is sufficient to promote Cdk2 phosphorylation on threonine (Fig. 4 A; and data not shown) and to activate the enzyme (Fig. 3 C). These results indicate that mechanisms other than increased cyclin E expression or cyclin E–Cdk2 complex assembly account for the inability of Ang II to induce threonine 160 phosphorylation of Cdk2 and to stimulate the activity of the enzyme in aortic SMC.



**Figure 3.** Regulation of CAK, Cdk4, and Cdk2 enzymatic activity by Ang II and PDGF-BB during G<sub>1</sub> progression. Quiescent aortic SMC were stimulated for different times with 100 nM Ang II (□) or 50 ng/ml PDGF-BB (■). Detergent lysates of the cells were prepared and equal amounts of proteins were subjected to immunoprecipitation with specific anti-Cdk2 (A), anti-Cdk4 (B), or anti-Cdk2 (C) antibodies. The phosphotransferase activity of the immunoprecipitates was assayed as described in Materials and Methods using GST-Cdk2K33M, GST-pRb, and histone H1 as substrates, respectively. The enzymatic activities are expressed as picomoles of phosphate incorporated into the substrate per min per mg of lysate protein. Similar results were obtained in three different experiments.

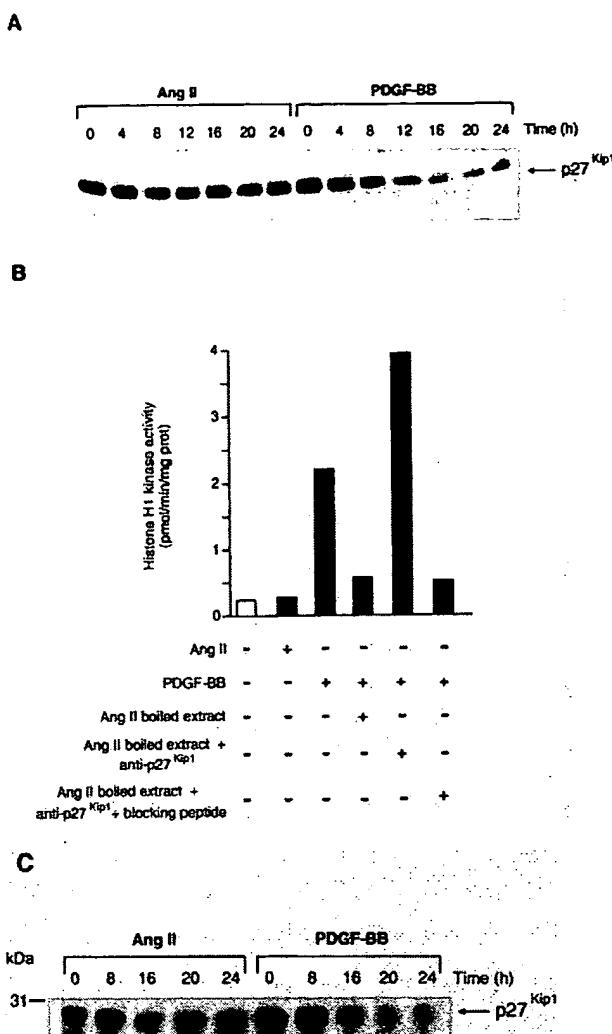
#### Differential Modulation of p27<sup>Kip1</sup> Expression by PDGF-BB and Ang II

In addition to its ability to disrupt the catalytic activity of phosphorylated cyclin-bound Cdk5 (Russo et al., 1996), the inhibitor p27<sup>Kip1</sup> can also sterically interfere with the phosphorylation of Cdk5 by CAK (Kato et al., 1994; Polyak et al., 1994a; Aprelikova et al., 1995). To determine whether p27<sup>Kip1</sup> was a determinant factor in the differential regulation of Cdk2 activation by mitogenic and hypertrophic factors, we compared the levels of p27<sup>Kip1</sup> protein expression. The expression of p27<sup>Kip1</sup> protein was elevated in quiescent aortic SMC and decreased progressively upon



**Figure 4.** Ang II fails to stimulate the phosphorylation of Cdk2. A, Phosphorylation of Cdk2. Quiescent aortic SMC were stimulated for 20 h with 100 nM Ang II or for 10 and 20 h with 50 ng/ml PDGF-BB and labeled with [<sup>32</sup>P]phosphoric acid during the last 5 h. Cell lysates were prepared and Cdk2 was immunoprecipitated using a specific antibody preadsorbed to protein A-Sepharose beads. The immunoprecipitated proteins were resolved by SDS-gel electrophoresis on 10% acrylamide gel, transferred to PVDF membrane, and analyzed by autoradiography. B, Phosphoamino acid analysis. The <sup>32</sup>P-labeled protein band corresponding to Cdk2 was excised from the PVDF membrane and subjected to partial acid hydrolysis. The phosphorylated amino acids were separated by one-dimensional thin layer electrophoresis. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated. C, Complex formation between Cdk2 and cyclin E. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Cell lysates were prepared and subjected to immunoprecipitation with cyclin E-specific antibody. The immunoprecipitated proteins were then analyzed by immunoblotting with anti-Cdk2 antibody. Similar results were obtained in three different experiments.

treatment of cells with the mitogenic factor PDGF-BB (Fig. 5 A). The decrease in p27<sup>Kip1</sup> level was already evident four hours after PDGF-BB exposure. After 20 h of stimulation with PDGF-BB, the expression of p27<sup>Kip1</sup> was reduced by ~80%. In contrast, Ang II had a negligible effect on the expression of the Cdk inhibitory protein. Importantly, we found that mixing of boiled extract from Ang II-stimulated cells with an equal amount of extract from cells treated for 20 h with PDGF-BB significantly reduced Cdk2-associated histone H1 kinase activity (Fig. 5 B). p27<sup>Kip1</sup> previously has been shown to be heat-stable (Polyak et al., 1994b), thus making it a good candidate for the inhibitory factor of Ang II-boiled extracts. Indeed, the Cdk2 inhibitory activity present in Ang II-treated cells was completely eliminated after immunodepletion of p27<sup>Kip1</sup> with a specific antibody (Fig. 5 B). Preincubation of the anti-p27<sup>Kip1</sup> antibody with a saturating amount of immunogenic peptide completely restored the Cdk2 inhibitory activity, confirming that p27<sup>Kip1</sup> is the major factor responsi-



**Figure 5.** Differential modulation of p27<sup>Kip1</sup> expression by Ang II and PDGF-BB. A, Expression of p27<sup>Kip1</sup> protein. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis on 12% acrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with anti-p27<sup>Kip1</sup> antibody and the proteins visualized by chemiluminescence detection. B, Inhibitory activity of Ang II extracts. Quiescent aortic SMC were stimulated for 20 h with 100 nM Ang II or 50 ng/ml PDGF-BB. Extracts from PDGF-BB-treated cells, which contain active Cdk2, were prepared and mixed with boiled extracts from Ang II-stimulated cells as described in Materials and Methods. After incubation for 90 min at 4°C, Cdk2 was immunoprecipitated and histone H1 kinase activity was determined. p27<sup>Kip1</sup> was immunodepleted from boiled extracts by incubation for 1 h at 4°C with anti-p27<sup>Kip1</sup> antibody. Specificity of p27<sup>Kip1</sup> immunodepletion was confirmed by preincubation of the antibody with excess immunogenic (blocking) peptide. The enzymatic activities are expressed as picomoles of phosphate incorporated into histone H1 per min per mg of lysate protein. C, Decreased association of p27<sup>Kip1</sup> with cyclin E-Cdk2 complexes in PDGF-BB-treated cells. Detergent lysates of aortic SMC stimulated for the indicated times with 100 nM Ang II or 50 ng/ml PDGF-BB were subjected to immunoprecipitation with anticyclin E antibody and analyzed by immunoblotting with antibody to p27<sup>Kip1</sup>. Similar results were obtained in three different experiments.

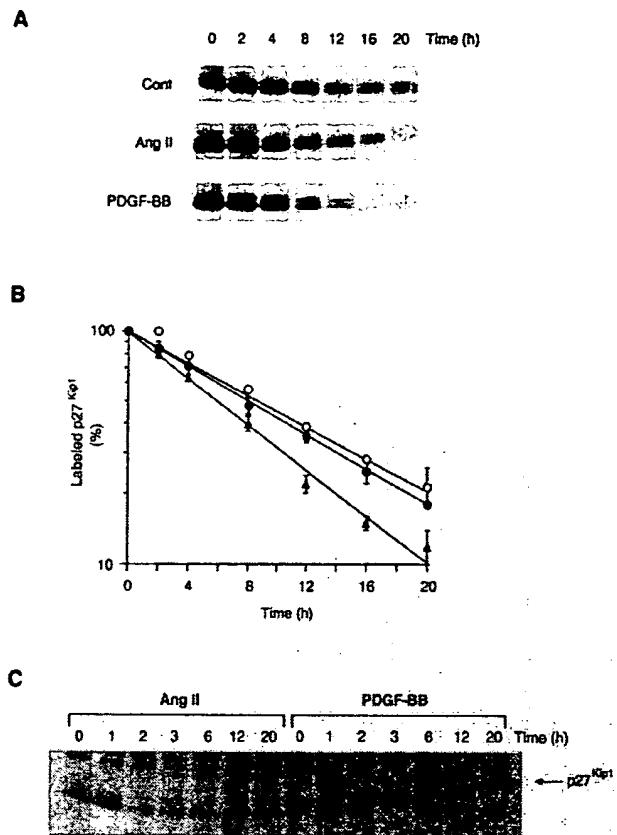
ble for this activity. Addition of boiled extracts from PDGF-BB-stimulated cells, which contain very low levels of p27<sup>Kip1</sup> (Fig. 5 A), did not inhibit Cdk2 activity of extracts from cells exposed to PDGF-BB for 20 h (data not shown).

The inability of Ang II to downregulate expression of p27<sup>Kip1</sup> in aortic SMC was also reflected by the strong and sustained association of the inhibitor with cyclin E-Cdk2 complexes. When cells exposed to Ang II or PDGF-BB were subjected to immunoprecipitation with anticyclin E antibody, the level of associated p27<sup>Kip1</sup> was found to be significantly lowered after 16 h of PDGF-BB treatment compared with Ang II-stimulated cells (Fig. 5 C). From these results, we conclude that the failure of Ang II to downregulate p27<sup>Kip1</sup> expression is responsible, at least in part, for the inability of Ang II to induce Cdk2 activation, DNA synthesis, and cellular division in aortic SMC.

### The Abundance of p27<sup>Kip1</sup> Is Regulated both at the Level of mRNA Expression and Protein Stability in Aortic SMC

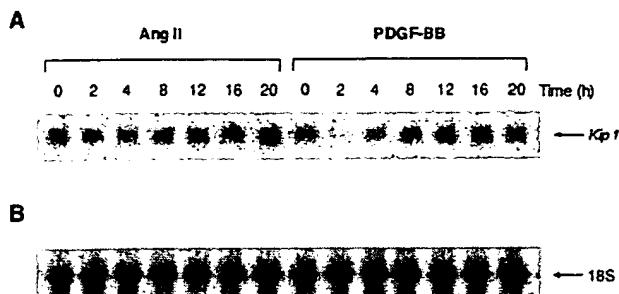
We next addressed the question of how the levels of p27<sup>Kip1</sup> are regulated by vascular growth factors. Studies in other cell systems have shown that the abundance of p27<sup>Kip1</sup> is controlled by multiple posttranscriptional processes including degradation through the ubiquitin-proteasome pathway (Pagano et al., 1995) and changes in translation rates (Agrawal et al., 1996; Hengst and Reed, 1996; Millard et al., 1997). To determine the rate of p27<sup>Kip1</sup> turnover, pulse-chase experiments were conducted on aortic SMC treated with Ang II or PDGF-BB. The rate of degradation of p27<sup>Kip1</sup> was clearly increased in cells exposed to PDGF-BB (Fig. 6, A and B). Quantitation of the data revealed that the half-life of the protein was reduced to six hours, compared with that of arrested (8.9 h) or Ang II-treated cells (8.2 h).

The rate of synthesis of p27<sup>Kip1</sup> was also affected by treatment with vascular growth factors. As shown in Fig. 6 C, the synthesis of p27<sup>Kip1</sup> was dramatically repressed after two hours of PDGF-BB stimulation and this inhibition persisted for up to 20 h. Ang II treatment also resulted in the repression of p27<sup>Kip1</sup> synthesis, but the effect was less marked and more transient, the rate of synthesis returning to basal level within 6–12 h of stimulation. To verify whether the decline in p27<sup>Kip1</sup> synthesis was associated with a decrease in *Kip1* mRNA accumulation, we measured the steady-state levels of *Kip1* mRNA by Northern hybridization. Results of these experiments clearly demonstrated that expression of *Kip1* mRNA is regulated in aortic SMC. PDGF-BB treatment resulted in a rapid and marked decrease of *Kip1* mRNA, which was almost undetectable by two hours of stimulation, and then slowly returned to its quiescent level at ~12 h (Fig. 7). Ang II also reduced expression of *Kip1* mRNA, but the effect was smaller in comparison to PDGF-BB. The time course of *Kip1* mRNA downregulation and reappearance correlated well with the transient decrease in the rate of p27<sup>Kip1</sup> synthesis seen after PDGF-BB and Ang II treatment (Fig. 6 C). This suggests that repression of p27<sup>Kip1</sup> synthesis by vascular growth factors is likely attributable, at least in part, to a corresponding decrease of *Kip1* mRNA abundance.



**Figure 6.** Downregulation of p27<sup>Kip1</sup> expression is associated with increased turnover of the protein and repression of its synthesis. **A.** Turnover of p27<sup>Kip1</sup> protein. Quiescent aortic SMC were pulse-labeled with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine for 60 min, after which the medium was changed with fresh medium alone or containing 100 nM Ang II or 50 ng/ml PDGF-BB. At different times, cell extracts were prepared and subjected to immunoprecipitation with anti-p27<sup>Kip1</sup> antibody. After extensive washing, the immunoprecipitated proteins were separated by electrophoresis on 12% acrylamide gel and analyzed by fluorography using a PhosphorImager. **B.** Densitometric analysis of the data shown in A. ○, Unstimulated cells ( $r = 0.958$ ); ●, Ang II-treated cells ( $r = 0.969$ ); ▲, PDGF-BB-treated cells ( $r = 0.950$ ). The data points represent mean  $\pm$  SEM of four separate experiments. **C.** Synthesis of p27<sup>Kip1</sup>. Quiescent aortic SMC were stimulated with Ang II or PDGF-BB for the times indicated and metabolically labeled with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine during the last 20 min of incubation. Preparation of cell extracts and immunoprecipitation were conducted as above. Similar results were obtained in four separate experiments.

There was a tight temporal relationship between the decrease in the rate of p27<sup>Kip1</sup> synthesis observed at two hours after stimulation, the increased rate of degradation of the protein, and the change in the total amount of p27<sup>Kip1</sup>. If we consider that the rate of p27<sup>Kip1</sup> synthesis is almost null after two to three hours of PDGF-BB treatment and that the half-life of the protein is approximately six hours (Fig. 6), the level of p27<sup>Kip1</sup> protein should be reduced by ~50% eight to nine hours after mitogenic stimulation. This estimation is consistent with the data presented in Fig. 5 A.

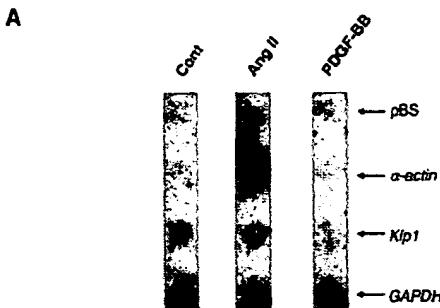


**Figure 7.** PDGF-BB produces a rapid and transient decrease of *Kip1* mRNA accumulation in aortic SMC. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for the indicated times. Total cellular RNA was extracted from the cells and analyzed by Northern hybridization using a  $^{32}$ P-labeled *Kip1* probe (A). The results were normalized by rehybridization of the blot with a 18S ribosomal RNA oligonucleotide probe (B). Similar results were obtained in five different experiments.

### PDGF-BB Reduces the Rate of *Kip1* Gene Transcription in Aortic SMC

To determine whether PDGF-BB-mediated downregulation of *Kip1* mRNA involves a transcriptional mechanism, nuclear run-on transcription assays were performed on nuclei isolated from quiescent and growth factor-treated aortic SMC. Fig. 8 shows that PDGF-BB markedly decreased the rate of *Kip1* transcription (~90% reduction of control value) after two hours of stimulation. Addition of Ang II also caused a significant attenuation of *Kip1* transcription, but the effect was less pronounced than that of PDGF-BB. As a control, we also examined transcription of the gene encoding smooth muscle  $\alpha$ -actin, which is known to be induced by Ang II, but not PDGF-BB, in vascular SMC (Corjay et al., 1990; Hautmann et al., 1997). In agreement with these studies, only Ang II enhanced smooth muscle  $\alpha$ -actin transcription. No appreciable difference in the transcription of *GAPDH* gene was observed in response to Ang II or PDGF-BB treatment.

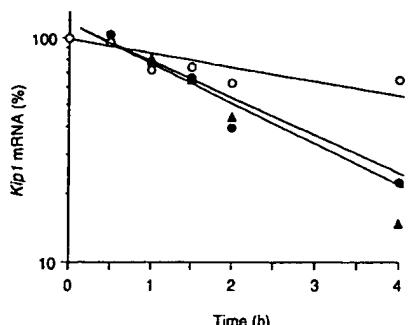
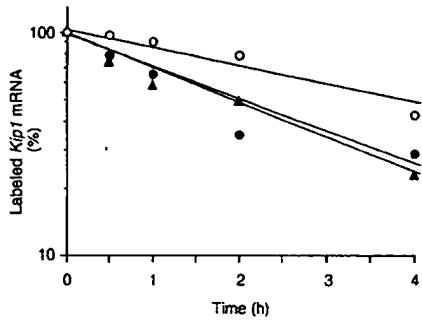
We next examined the effect of PDGF-BB and Ang II on the stability of *Kip1* mRNA using two independent approaches: mRNA decay in the presence of the transcriptional inhibitor 5,6-Dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) and pulse-chase analysis with  $^{3}H$ juridine. With the use of DRB, the half-life of *Kip1* mRNA was estimated to be 4.7 h in unstimulated cells (Fig. 9 A). Treatment with PDGF-BB or Ang II accelerated the degradation of *Kip1* mRNA, decreasing the half-life to 2.2 h and 2.3 h, respectively. Comparable results were obtained with the pulse-chase method with a calculated half-life of 3.6 h in quiescent cells, and of 1.9 h and 2.0 h in cells stimulated with PDGF-BB and Ang II, respectively (Fig. 9 B). These findings indicate that both PDGF-BB and Ang II destabilize *Kip1* mRNA to the same extent in aortic SMC. We found that the calculated half-life values of *Kip1* mRNA are slightly longer than what would be expected from the results of Fig. 7. This is likely due to the inherent imprecision associated with the measure of low abundant messages with short half-lives (Harrold et al., 1991).



**Figure 8.** PDGF-BB reduces transcription of *Kip1* gene in aortic SMC. A, Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for 2 h. Nuclei were isolated and the  $^{32}$ P-labeled nascent transcripts were hybridized to the indicated plasmid DNAs as described in Materials and Methods. The plasmid DNA inserts used in this experiment were pBluescript-SK vector (pBS), smooth muscle  $\alpha$ -actin ( $\alpha$ -actin), *Kip1*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, Densitometric analysis of the data shown in A expressed as the ratio *Kip1*/GAPDH. Similar results were obtained in two different experiments.

### The Failure of Ang II to Promote S Phase Entry of Aortic SMC Is Not Explained by Autocrine Production of TGF- $\beta$ 1

TGF- $\beta$ 1 is the prototype of a family of growth factors that play important roles in cellular growth, differentiation, and morphogenesis (Massague, 1990). In particular, TGF- $\beta$ 1 is a potent growth inhibitor for many cell types, including vascular SMC (Owens et al., 1988). Several mechanisms have been proposed to explain how TGF- $\beta$ 1 inhibits proliferation and induces cell cycle arrest in  $G_1$  phase (Hannon and Beach, 1994; Reynisdottir et al., 1995). The observation that Ang II can induce hypertrophic or mitogenic effects in vascular SMC has led to the hypothesis that Ang II activates both proliferative and antiproliferative, specifically TGF- $\beta$ 1, signals (Gibbons et al., 1992). Therefore, we carried out a series of experiments to test the possibility that autocrine production of TGF- $\beta$ 1 may be responsible for the failure of Ang II to activate Cdk2 and induce DNA synthesis in aortic SMC. To determine whether Ang II stimulates the production of active TGF- $\beta$ 1, we used a highly sensitive bioassay that is based on the

**A****B**

**Figure 9.** Effect of Ang II and PDGF-BB on the stability of *Kip1* mRNA. A, DRB method. Quiescent aortic SMC were either left untreated or stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for the indicated times in the presence of 25  $\mu$ g/ml DRB. Total cellular RNA was extracted from the cells and analyzed by Northern hybridization using a  $^{32}$ P-labeled *Kip1* probe. The hybridization signals were quantified using a PhosphorImager apparatus. ○, Unstimulated cells; ●, Ang II-treated cells; ▲, PDGF-BB-treated cells. Data points represent the mean of three separate experiments. B, Pulse-chase method. Quiescent aortic SMC were pulse-labeled with [ $^3$ H]uridine for 12 h and chased with unlabeled uridine and cytidine in the presence or absence of Ang II or PDGF-BB. The effective chase was started 2 h after addition of cold nucleosides (zero time point). At different times, total  $^3$ H-labeled RNA was isolated and hybridized to *Kip1* cDNA immobilized onto nitrocellulose filters. Data points are the mean of two separate experiments. For each method, the values are expressed as percentage of time zero. The solid lines represent the least-squares fit of the data obtained by linear regression analysis.

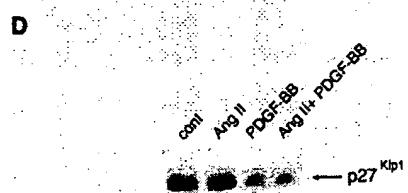
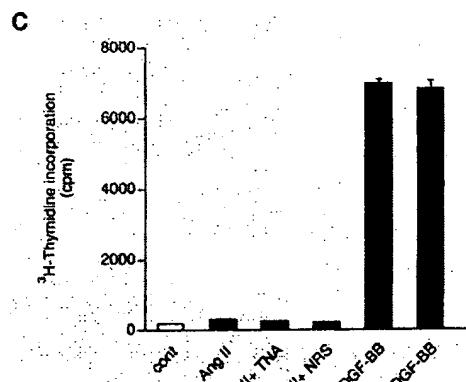
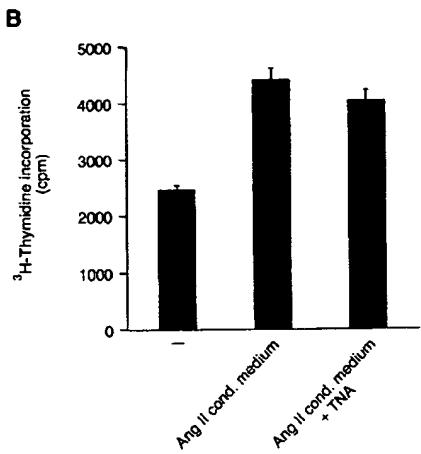
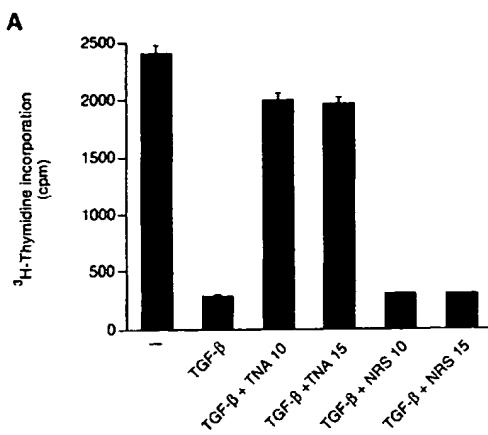
ability of TGF- $\beta$  to induce  $G_1$  arrest in Mv1Lu cells. Fig. 10 A shows that addition of 10 pM TGF- $\beta$ 1 to Mv1Lu cells is sufficient to inhibit DNA synthesis by 90%. This inhibitory activity of TGF- $\beta$ 1 is reversed by coincubation with TNA, but not with normal rabbit IgG. However, conditioned medium from Ang II-treated aortic SMC did not inhibit Mv1Lu cells DNA synthesis, but rather had a significant stimulatory effect (Fig. 10 B). We also tested the effect of TNA on the ability of Ang II to stimulate DNA synthesis in aortic SMC. Coincubation of Ang II with normal rabbit IgG or TNA had essentially no effect on the rate of DNA synthesis (Fig. 10 C). Finally, we examined the effect of simultaneous exposure of aortic SMC to both PDGF-BB and Ang II. Simultaneous addition of Ang II or pretreatment with Ang II (data not shown) did not inter-

fer with PDGF-BB-induced DNA synthesis (Fig. 10 C) or p27 $^{kip1}$  downregulation (Fig. 10 D), consistent with the idea that Ang II does not stimulate the synthesis of an antimitogenic factor. Together, these results demonstrate that the failure of Ang II to promote S phase entry of aortic SMC is not due to autocrine production of TGF- $\beta$ 1.

## Discussion

Unlike cardiac and skeletal muscle cells, which undergo terminal and irreversible differentiation, vascular SMC display remarkable cellular plasticity that allows them to acquire a spectrum of different phenotypes in response to appropriate stimuli (Owens, 1995). In addition to their main function of contraction, vascular SMC can increase their mass through cellular proliferation, cellular hypertrophy, and production of extracellular matrix proteins. Changes in growth rates occur normally during development of the vascular system and after vascular injury, but also under pathological conditions such as hypertension and atherosclerosis (Schwartz et al., 1986; Owens, 1989; Ross, 1993). In animal models of hypertension, the increase in vascular mass is associated primarily with SMC hypertrophy in large arteries and with hyperplasia in small resistance vessels. SMC proliferation also plays a central role in the atherosclerotic process. The growth response of vascular SMC is clearly dependent on the nature of the growth stimulus. For example, in cultured rat aortic SMC, agonists like Ang II induce cellular hypertrophy as a result of increased protein synthesis (Geisterer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Giasson and Meloche, 1995), whereas peptide growth factors like PDGF-BB cause a strong proliferative response (Raines et al., 1990; Grainger et al., 1994). However, much remains to be learned about the molecular determinants of vascular SMC hypertrophic versus hyperplastic growth response. Here, we present evidence that p27 $^{kip1}$  is an important regulator of the phenotypic response of vascular SMC. First, we show that treatment with the mitogenic factor PDGF-BB, but not with the hypertrophic factor Ang II, leads to a progressive and dramatic decline in the level of p27 $^{kip1}$  protein. The failure of Ang II to downregulate p27 $^{kip1}$  results in the increased association of the inhibitor with cyclin E-Cdk2 complexes and correlates with inhibition of threonine 160 phosphorylation of Cdk2. Since CAK is constitutively active in aortic SMC, the simplest interpretation of our data is that stoichiometric binding of p27 $^{kip1}$  to cyclin E-Cdk2 complexes prevents CAK from phosphorylating and activating Cdk2 in Ang II-treated cells. In support of this hypothesis, in vitro studies have shown that p27 $^{kip1}$  binding to preformed cyclin E-Cdk2 complexes blocks CAK-mediated threonine 160 phosphorylation of the enzyme (Polyak et al., 1994a; Aprelikova et al., 1995). Second, we further show that extracts from Ang II-stimulated cells contain enough CDK inhibitory activity to reduce by ~70% Cdk2-associated histone H1 kinase activity of PDGF-BB-treated cell extracts. The stability of this inhibitory activity to heat treatment and its reversal following immunodepletion of p27 $^{kip1}$  confirmed that p27 $^{kip1}$  is the major inhibitory factor present in these extracts.

Previous studies have shown that the abundance of



**Figure 10.** Ang II does not stimulate autocrine production of TGF- $\beta$ 1 in aortic SMC. A, TGF- $\beta$ 1 bioassay. Mv1Lu cells were treated for 24 h with 10 pM TGF- $\beta$ 1 in the absence or presence of 10–15  $\mu$ g/ml of TNA or normal rabbit IgG (NRS). B, Conditioned medium from Ang II-stimulated aortic SMC (1:5 dilution)

p27 $^{Kip1}$  is regulated by multiple posttranscriptional mechanisms. Our present results add another level of complexity by demonstrating that the levels of p27 $^{Kip1}$  are also controlled by transcriptional mechanisms in vascular SMC. Our data support a model where the reduction of p27 $^{Kip1}$  expression observed in response to mitogenic factors occurs by two mechanisms. The first mechanism is a rapid decrease in the rate of p27 $^{Kip1}$  synthesis that becomes minimal by two hours and slowly returns to quiescent value after  $\sim$ 20 h. This lowered synthesis, combined with the significant turnover of the protein (see Fig. 6 B), is responsible for the initial decline in p27 $^{Kip1}$  protein levels, which can be easily detected by eight hours of PDGF-BB stimulation. Detailed kinetic analysis revealed that the reduction in the rate of p27 $^{Kip1}$  synthesis is tightly paralleled by a transient downregulation of *Kip1* mRNA accumulation. Importantly, these changes in *Kip1* mRNA levels coincide with a marked decrease in the rate of *Kip1* gene transcription, suggesting that transcriptional control is an important factor in regulating the synthesis of p27 $^{Kip1}$ . While other studies have reported changes in the levels of *Kip1* mRNA in response to extracellular factors (Kwon et al., 1996; Liu et al., 1996), our findings provide the first demonstration that p27 $^{Kip1}$  expression is regulated at the level of gene transcription. We also show that both PDGF-BB and Ang II significantly decrease the stability of *Kip1* mRNA. The almost complete inhibition of *Kip1* gene transcription, coupled with the increased turnover of the mRNA, explains the marked downregulation of *Kip1* mRNA expression observed in PDGF-BB-treated cells. Further studies are clearly necessary to identify the cis-acting elements that target *Kip1* mRNA for degradation and the corresponding RNA-binding proteins. In addition to transcription, other levels of control may also be involved in the regulation of p27 $^{Kip1}$  synthesis. Figs. 6 C and 7 show that the rate of p27 $^{Kip1}$  synthesis is still repressed in PDGF-BB-treated cells after 12–20 h when *Kip1* mRNA has returned to control levels. One possibility is that *Kip1* mRNA is not being used efficiently by the translation machinery during G<sub>1</sub> progression because of the binding of mRNA masking proteins (Spirin, 1996). In support of this idea, it was found that the accumulation of p27 $^{Kip1}$  protein observed during growth arrest of HL-60 cells is due to an increase in the amount of *Kip1* mRNA in polyribosomes (Millard et al., 1997). The second mechanism of p27 $^{Kip1}$  elimination is an increase in the degradation rate of the protein, which is mostly evident by eight hours of mitogenic stimulation. By contrast, treatment of vascular SMC

was added alone or in combination with 10  $\mu$ g/ml TNA to Mv1Lu cells. C, Quiescent aortic SMC were stimulated for 24 h with 100 nM Ang II in the absence or presence of 10  $\mu$ g/ml TNA or NRS. In the same experiment, the cells were stimulated with 50 ng/ml PDGF-BB alone or in the presence of 100 nM Ang II. The rate of DNA synthesis was measured by [<sup>3</sup>H]thymidine incorporation during the last 4–6 h of stimulation. Each value represents the mean  $\pm$  SEM of triplicate determinations. The data presented are representative of at least two different experiments with similar results. D, Expression of p27 $^{Kip1}$  protein. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB or with both agonists for a 20-h period. The expression of p27 $^{Kip1}$  protein was analyzed by immunoblotting.

with hypertrophic factors like Ang II less effectively represses p27<sup>Kip1</sup> synthesis and does not affect the rate of degradation of the protein.

The signaling pathways that are involved in the regulation of *Kip1* gene transcription remain to be identified. As mentioned earlier, PDGF-BB and Ang II activate several common signaling events in aortic SMC. However, significant differences are noted in the time course of these events. For example, PDGF-BB induces a sustained activation of the MAP kinases ERK1/ERK2, whereas Ang II has a very transient effect (Plevin et al., 1996; and data not shown). PDGF-BB and Ang II are also known to have different effects on the source and duration of the increase in cytosolic-free calcium in vascular SMC (Roe et al., 1989; Brinson et al., 1998). In addition, mitogenic and hypertrophic factors are likely to trigger unique signaling events. Studies in pulmonary arterial SMC have shown that PDGF-BB exclusively stimulates an increase in phosphatidylinositol 3,4,5-trisphosphate (Button et al., 1994), whereas only thrombin, which behaves as a hypertrophic factor, induces *fosB* mRNA levels (Rothman et al., 1994). However, these observations may not be generalized to other SMC types, since both PDGF-BB and Ang II activate PI3-kinase and induce *fosB* mRNA in rat aortic SMC (Saward and Zahradka, 1997; and data not shown). Characterization of the 5' flanking region of the mouse *Kip1* gene showed that a region between -326 to -615 is sufficient to confer maximal basal promoter activity (Kwon et al., 1996; Zhang and Lin, 1997). Constructs extending beyond -615 displayed lower basal promoter activity, suggesting that a negative regulatory element may be contained in the region between -615 and -1,609 (Kwon et al., 1996). However, these studies did not examine the serum or growth factor responsiveness of the various *Kip1* gene promoter constructs. Work is in progress in our laboratory to identify specific regions within the promoter of the rat *Kip1* gene which mediate PDGF-BB dependent transcriptional repression.

The turnover of p27<sup>Kip1</sup> is also subject to regulation by mitogenic factors in vascular SMC. Given the recent demonstration that cyclin E-Cdk2 directly phosphorylates p27<sup>Kip1</sup> on threonine 187 and promotes its elimination from the cell (Sheaff et al., 1997; Vlach et al., 1997), it is tempting to speculate that the different rates of p27<sup>Kip1</sup> turnover observed in PDGF-BB or Ang II-treated cells are a reflection of their differential ability to activate Cdk2. In agreement of this idea, we found that *in vivo* phosphorylation of p27<sup>Kip1</sup> increases after 8–12 h in cells exposed to PDGF-BB, but not in response to Ang II (data not shown). However, phosphorylation by Cdk2 is unlikely to be the sole mechanism that regulates the proteolysis of p27<sup>Kip1</sup>. Indeed, significant degradation of the inhibitor is observed during the first hours of growth factor stimulation, in the absence of detectable histone H1 kinase activity (Pagano et al., 1995; Agrawal et al., 1996; this study). We also found that in vascular SMC and other cell types, p27<sup>Kip1</sup> is significantly phosphorylated in G<sub>0</sub> and early G<sub>1</sub> phase (data not shown). These observations suggest that other protein kinases and/or mechanisms signal p27<sup>Kip1</sup> for degradation. In this respect, it was reported that Ras signaling is required for downregulation of p27<sup>Kip1</sup> in rodent fibroblasts (Aktas et al., 1997; Takuwa and Takuwa, 1997;

Kawada et al., 1997) and that RhoA is a necessary mediator of p27<sup>Kip1</sup> degradation (Weber et al., 1997).

It has been postulated that the failure of Ang II to stimulate vascular SMC hyperplasia is due to autocrine production of the antimitogenic cytokine TGF- $\beta$ 1 by these cells (Gibbons et al., 1992; Koibuchi et al., 1993). However, our results do not support this model. First, active TGF- $\beta$ 1 was not detected in the supernatant of Ang II-treated aortic SMC. Second, the use of a neutralizing antibody against TGF- $\beta$ 1 in combination with Ang II did not potentiate DNA synthesis in these cells. Third, pretreatment of aortic SMC for four hours with Ang II before PDGF-BB stimulation (data not shown) or simultaneous addition of both factors did not affect the mitogenic response to PDGF-BB.

Previous *in vivo* studies have demonstrated that Cdk2 function is required for intimal SMC accumulation after angioplasty in the rat carotid artery (Abe et al., 1994; Morishita et al., 1994). In addition, Cdk2 expression is temporally correlated with vascular SMC proliferation after angioplasty (Wei et al., 1997). More recently, it was reported that p27<sup>Kip1</sup> is markedly upregulated after balloon angioplasty in the rat carotid artery and that high levels of p27<sup>Kip1</sup> expression correlates with downregulation of Cdk2 kinase activity (Chen et al. 1997). Ectopic overexpression of p27<sup>Kip1</sup> in injured arteries attenuated neointimal lesion formation. A recent study also presented evidence that polymerized collagen inhibits aortic SMC proliferation *in vitro* through  $\alpha$ 2 integrin-mediated upregulation of p27<sup>Kip1</sup> (Koyama et al., 1996). Thus, the results presented here, together with these findings, clearly identify p27<sup>Kip1</sup> as an important regulator of vascular SMC growth response.

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# Inhibition of Retinoblastoma Protein Phosphorylation by Myogenesis-induced Changes in the Subunit Composition of the Cyclin-dependent Kinase 4 Complex<sup>1</sup>

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## Abstract

The retinoblastoma protein (Rb) is essential for the maintenance of the postmitotic state in terminally differentiated myocytes. Upon C2C12 myogenesis, the level of the cyclin-dependent kinase 4 (CDK4) protein does not change, but its Rb kinase activity is down-regulated markedly. Here, we show that the reduction in CDK4 activity results from (a) the irreversible induction and association of the p21 CDK inhibitor with the CDK4 complex and (b) a decline in overall D-type cyclin expression. Immunoprecipitation-coupled immunoblot analyses demonstrated that myocyte differentiation produces alterations in the subunit interactions within the CDK4 complex, including a diminished interaction with cyclin D1 and enhanced interactions with cyclin D3 and p21. The significance of the p21 interaction with CDK4 was indicated by the ability of anti-p21 antibodies to specifically immunodeplete a Rb kinase inhibitory activity that was bound to the CDK4 complex in myotubes. Furthermore, the restimulation of myotubes with serum did not lead to the re-activation of CDK4 or disrupt the CDK4-p21 interaction. Despite the increase in cyclin D3 expression during myogenesis, quantitative immunoblot analyses revealed that the combined levels of cyclin D1 and D3 decline during this process and that CDK4 is expressed at much higher levels than either of these cyclin subunits in postmitotic myotubes. These results suggest that the myogenesis-induced up-regulation of p21 and down-regulation of the total D-type cyclin expression contribute to the inhibition of the CDK4 Rb kinase activity, leading to conditions that favor the accumulation of the hypophosphorylated Rb and growth arrest upon terminal differentiation.

## Introduction

During skeletal muscle terminal differentiation, the postmitotic state is established prior to the expression of the contractile phenotype (1). The Rb<sup>3</sup> appears to be an important regulator of the cell cycle withdrawal during myogenesis. Myogenic differentiation is associated with an increase in the steady-state level of Rb mRNA (2) and the accumulation of the hypophosphorylated (active) form of the Rb protein (3, 4). Myocytes derived from a Rb<sup>-/-</sup> mouse can differentiate into multinucleated myotubes, but unlike wild-type cells, they remain capable of cell cycle reentry upon mitogen restimulation (5). In undifferentiated cells, Rb is hypophosphorylated in the quiescent state and in the early G<sub>1</sub> phase of the cell cycle, where it blocks cell cycle progression because of the inactivation of the E2F transcription factor (6). The phosphorylation of Rb is initiated during mid- to late G<sub>1</sub>, and it becomes fully phosphorylated prior to G<sub>1</sub>-S transition. Subsequently, it is dephosphorylated during mitosis. CDK4 and CDK6 are thought to be the predominant Rb kinases *in vivo* (7). The cyclin A-CDK2 and cyclin E-CDK2 complexes can also phosphorylate Rb *in vitro* and promote Rb hyperphosphorylation in transfected cells (8). CDK2 activation by cyclin E occurs following the initial phosphorylation of Rb, and the activation of CDK2 by cyclin A occurs at even later time points. In contrast, activation of cyclin D-CDK complexes occur in the mid-G<sub>1</sub> phase (9, 10), the same phase of the cell cycle in which myoblasts commit to terminal differentiation (11-13).

Although Rb becomes dephosphorylated during myocyte differentiation, the predominant Rb kinases, CDK4 and CDK6, are constitutively expressed (14-16). It has also been reported that cyclin D3 is up-regulated 20-fold during L<sub>6</sub> cell myogenic differentiation, but no cyclin D3-associated Rb kinase activity could be detected in lysates prepared from the differentiated myotube cultures (15). Thus, the mechanisms leading to Rb dephosphorylation upon myogenesis are not obvious, and they likely involve complex changes in the subunit compositions of the CDK4 and CDK6 complexes. Recently, cyclin kinase inhibitors were identified that bind to the CDK complexes and function as negative regulators of cell growth. The general CDK inhibitor p21 (also referred to as sdi1, mda-20, CIP1, and WAF1) is induced markedly upon skeletal muscle differentiation (1, 16-18). p21 functions to inhibit CDK2 activity in postmitotic myotubes (18), which presumably contributes to the establishment of the postmitotic state, and its expression also serves to promote myocyte survival in low mitogen differentiation medium (19). Here we examined the regulation of the CDK4 kinase

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<sup>3</sup> The abbreviations used are: Rb, retinoblastoma protein; CDK, cyclin-dependent kinase; GST, glutathione S-transferase.

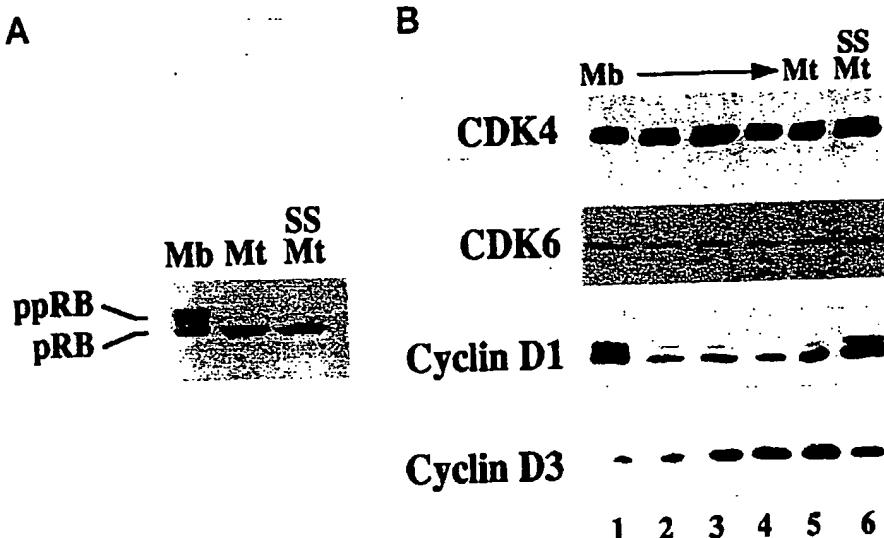


Fig. 1. Rb phosphorylation and profiles of cyclin and CDK protein expression during myogenic differentiation. A, immunoblot analysis of Rb in C2C12 myoblasts (Mb), myotubes (Mt), and myotubes cultured in growth media for 24 h (SS). The hypophosphorylated form (pRb) and hyperphosphorylated form (ppRb) of Rb proteins are indicated. B, profile of cyclin and CDK protein expression during myogenesis. Levels of CDK4, CDK6, cyclin D1, and cyclin D3 during C2C12 cell differentiation were monitored by immunoblot analysis. Lane 1, myoblasts; Lanes 2–4, myoblasts transferred to differentiation media for 24, 48, and 72 h, respectively; Lane 5, myotubes that were prepared by incubating confluent cultures of myoblasts in differentiation media for 5 days and treating with  $\beta$ -D-arabinofuranoside (see "Materials and Methods"); Lane 6, cultures of myotubes transferred to growth media for 24 h. Cyclin D2 was undetectable in C2C12 myocytes under conditions employed here (data not shown).

activity during myogenesis. We found that despite the constitutive expression of the CDK4 protein, its kinase activity was reduced markedly during C2C12 myocyte differentiation. Our data suggest that myogenesis-induced changes in the subunit composition of the CDK4 complex contribute to this inhibition of CDK4 kinase activity. These changes result from the up-regulation of the p21 CDK inhibitor and from a decrease in the combined levels of the D-type cyclins during terminal differentiation.

## Results

**Changes in Rb Phosphorylation and CDK4 Rb Kinase Activity upon Myocyte Differentiation.** Immunoblotting analyses were performed to monitor changes in Rb phosphorylation during C2C12 differentiation under the cell culture conditions employed for these studies (Fig. 1A). As reported previously (3, 4), both the rapidly migrating hypophosphorylated forms and the slowly migrating hyperphosphorylated forms of Rb are present in replicating myoblast lysates. However, the hypophosphorylated form predominates in lysates prepared from myotube cultures and in lysates prepared from postmitotic myotube cultures that were restimulated with the mitogen-rich growth media. We next examined the levels of D-type cyclins and their associated kinases, CDK4 and CDK6, which are the predominant Rb kinases *in vivo*. CDK4 and CDK6 protein levels remained constant during myocyte differentiation (Fig. 1B). Cyclin D1 protein levels decreased, whereas cyclin D3 protein levels increased 3–4-fold. Cyclin D2 was not detectable in the C2C12 myotube or myoblast lysates, but it has been reported that cyclin D2 protein levels decline during L<sub>6</sub> myocyte differ-

entiation (15). The changes in D1 and D3 cyclin levels are consistent with previously reported changes in their mRNA and protein levels during myocyte differentiation (14–16). Serum restimulation of differentiated myotubes had no effects on the levels of CDK4 or CDK6 but appeared to partially reverse the myogenesis-induced changes in cyclin D1 and cyclin D3 levels. Because the myotube cultures used for these experiments comprised greater than 90% of the nuclei in multinucleated cells, these data suggest that the myogenesis-induced changes in the expression cyclins D1 and D3 are reversible, and they are consistent with the notion that the mitogenic signal transduction pathway is intact in postmitotic myotubes (18, 20, 21). Because the CDK6 band was of low intensity in the immunoblot (Fig. 1B) and CDK6 immunoprecipitates displayed a very low Rb kinase activity under our assay conditions (not shown), we focused on CDK4 and its associated proteins in subsequent experiments.

To investigate the mechanisms that may contribute to the observed changes in Rb phosphorylation during C2C12 myogenesis, the *in vitro* Rb kinase activities of CDK4, cyclin D1, and cyclin D3 immunoprecipitates were determined using a GST-Rb fusion protein as substrate. Relatively high levels of Rb kinase activities were detected in all immunoprecipitates from myoblast cell lysates, whereas these Rb kinase activities were considerably lower in cell lysates prepared from myotubes and myotubes restimulated with serum (Fig. 2). Similarly, a reduction in cyclin D3 and CDK4-associated Rb kinase activity during L<sub>6</sub> myocyte differentiation has also been observed (15). This reduction of *in vitro* Rb kinase activity is consistent with the observed changes in Rb phosphorylation states during myo-

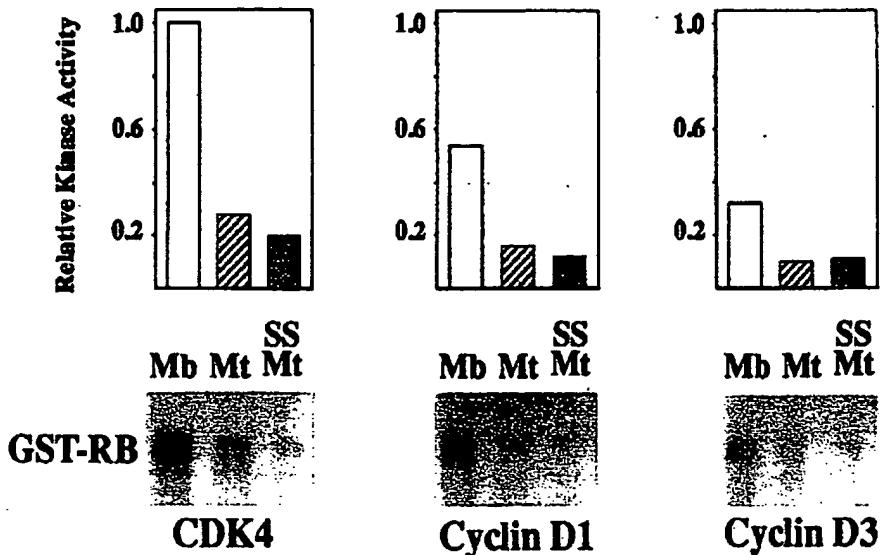


Fig. 2. Down-regulation of Rb kinase activity during myogenic differentiation. Anti-CDK4, cyclin D1, and cyclin D3 immunoprecipitates from lysates of myoblasts (Mb; □), myotubes (Mt; ▨), or myotubes restimulated with serum (SSMt; ■) were collected on protein A plus G beads. The Rb kinase activities of these protein complexes were measured using a GST-Rb fusion protein as substrate (see "Materials and Methods"). Kinase assays were performed simultaneously, and the intensities of the autoradiography bands can be compared directly. The average values from two independent experiments are shown in the histogram.

genesis. However, it is paradoxical that the level of CDK4 remains constant and that the level of cyclin D3 is up-regulated during this process (Fig. 1B), whereas the CDK4/cyclin D3 kinase activity is down-regulated in myotubes. It is also paradoxical that the cyclin D1-associated Rb kinase activity remains low in myotubes that are restimulated with serum, despite the reinduction of cyclin D1 expression to levels comparable to that in proliferating myoblasts. These data led us to investigate the regulation of CDK4 regulatory subunits in greater detail.

**Relative Levels of CDK4, Cyclin D1, Cyclin D3, and p21.** Because of differences in antibody efficiency, direct comparisons between cell cycle protein levels cannot be made based solely on the data presented in Fig. 1B. However, relative levels of cell cycle proteins could be estimated by comparing immunoblot band intensities after they were calibrated relative to the band intensities of known amounts of recombinant proteins that were simultaneously processed during the immunoblot procedure (Fig. 3). On the basis of these analyses, the calculated levels of CDK4 and p21 in myotube lysates are 20 and 31 fmol/μg of cell lysate, respectively. In contrast, the levels of cyclin D1 and cyclin D3 are significantly lower in myotube lysates: 1 and 3 fmol/μg of cell lysate, respectively. These estimates of cyclin and CDK4 levels in myotubes indicate that the majority of CDK4 is not complexed with cyclins in myotubes, and that the total amount of p21 greatly exceeds the levels of the active CDK4/cyclin complexes in these cells. In contrast, the levels of cyclin D1 in myoblast lysates are 10 fmol/μg of cell lysate (not shown). Thus, the large decline in cyclin D1 levels is only partially compensated by the increase in cyclin D3 expression, and this situation is likely to contribute to the low levels of CDK4 activity that are observed in myotube lysates (Fig. 2).

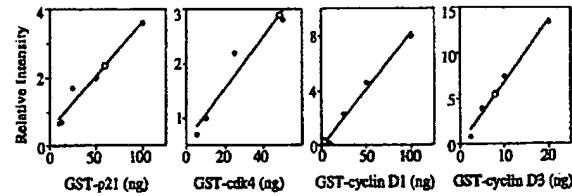


Fig. 3. Estimates of p21, CDK4, cyclin D1, and cyclin D3 levels in myotube lysates. Immunoblot analyses were performed simultaneously with specific antibodies on 40 μg of myotube lysate and on the indicated quantities of recombinant GST fusion protein. Band intensities were determined by scanning densitometry and plotted. The quantity of the specific protein in the myotube lysate was estimated from its band intensity (○) relative to the band intensities of known quantities of recombinant protein (●).

**Myogenesis-induced Changes in CDK4-associated Proteins.** CDK4-associated proteins were analyzed by coimmunoprecipitation using lysates prepared from [<sup>35</sup>S]methionine-labeled cultures of myoblasts and myotubes to further investigate the mechanism of the CDK4 Rb kinase inhibition (Fig. 4). Immunoprecipitation of C2C12 myoblast lysates with anti-CDK4 antibodies revealed predominant bands that appear to correspond to p32<sup>CDK4</sup> and p32<sup>cyclinD3</sup>, which comigrate in SDS-PAGE, and p35<sup>cyclinD1</sup>. Several lower-intensity bands were also detected that had apparent molecular weights of  $M_r$  16,000, 21,000, 26,000, and 27,000 (Fig. 4, Lane 1). The immunoprecipitates of the corresponding myotube lysates displayed a reduction in the relative intensity of the putative p35<sup>cyclinD1</sup> band and an increase in the intensities of the  $M_r$  21,000, 26,000, and 27,000 bands (Fig. 4, Lane 2). The specificity of the immunoprecipitated bands was demonstrated by their competition with an excess of immunogenic CDK4

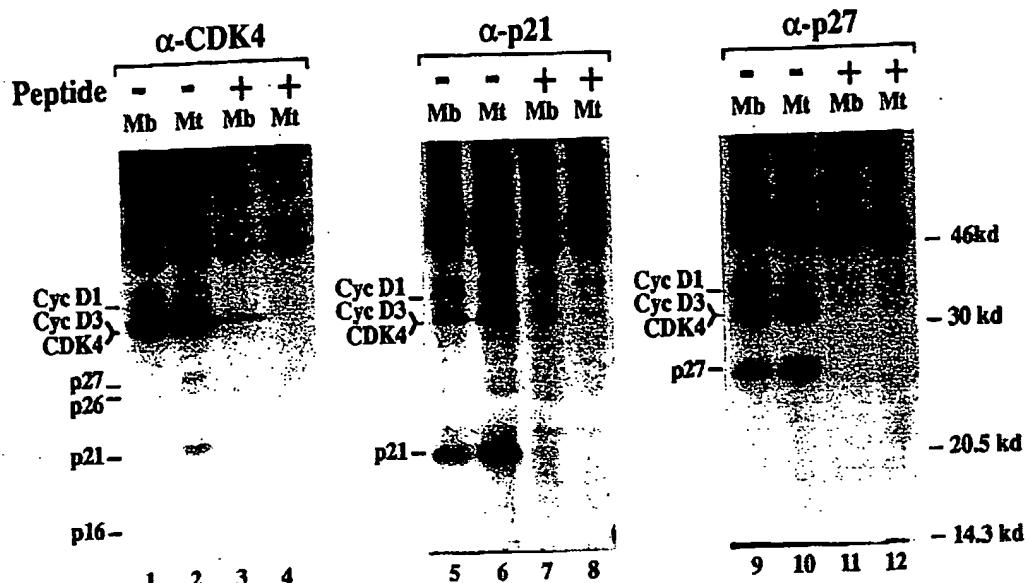


Fig. 4. Immunoprecipitation analyses of CDK4 complexes in myoblast and myotube lysates. [<sup>35</sup>S]methionine-labeled cell lysates from myoblast (Mb) and myotube (Mt) cultures were immunoprecipitated with anti-CDK4 (Lanes 1-4), anti-p21 (Lanes 5-8), and anti-p27 (Lanes 9-12) antibodies. The precipitated protein complexes were separated on SDS-PAGE gel, and the [<sup>35</sup>S]-labeled protein bands were visualized by fluorography. Parallel immunoprecipitation reactions were performed in the presence of competitive peptides to demonstrate the specificity of the immunoprecipitation. Positions of the putative p35<sup>cyclinD1</sup>; p32<sup>cyclinD3/CDK4</sup>, and the M<sub>r</sub> 16,000; 21,000, 26,000, and 27,000 bands are indicated. The three gels were run independently, and the molecular weight markers are shown for only one of them.

peptide (Fig. 4, Lanes 3 and 4). The myogenesis-inducible  $M_r$  21,000 and 27,000 proteins in the CDK4 immunoprecipitates had electrophoretic mobilities similar to that of the cyclin kinase inhibitors p21 and p27 (KIP1). Thus, immunoprecipitation analyses were also performed with anti-p21 (Fig. 4, Lanes 5-8) and anti-p27 (Fig. 4, Lanes 9-12) antibodies. These analyses revealed similar sets of <sup>35</sup>S-labeled bands corresponding to p21 or p27 as well as their associated proteins, including p32<sup>cyclinD4</sup>, p32<sup>cyclinD3</sup> and p35<sup>cyclinD1</sup>.

A series of immunoprecipitation-coupled immunoblotting experiments were performed to confirm the identities of CDK4-associated proteins and more accurately assess the myogenesis-induced changes to their relative levels (Fig. 5). Consistent with data from the simple immunoblot analyses (Fig. 1), the immunoprecipitation-coupled immunoblots revealed no change in the total level of CDK4, a decrease in the level of cyclin D1, and increases in the level of cyclin D3. This analysis also revealed an increase in p21 expression. Analysis of CDK4 immunoprecipitates demonstrated enhanced associations of CDK4 with cyclin D3 (8-fold) and with p21 (10-fold) during myogenesis, whereas the level of CDK4-associated cyclin D1 decreased by a factor of 4 (Fig. 5; compare Lanes 6 and 7). These results were confirmed by using anti-CDK4 antibodies to immunoblot the anti-p21, anti-cyclin D1, and anti-cyclin D3 immunoprecipitates (Fig. 5, top row). Finally, these analyses did not detect cyclin D1 in cyclin D3 immunoprecipitates, and vice versa.

The immunoprecipitation-coupled immunoblot strategy was also employed to examine the interaction between p27 and CDK4 (Fig. 6). p27 was detected in CDK4 immunoprecipitates and CDK4 was detected in p27 immunoprecipi-

tates. Upon myogenesis, the levels of p27 increased approximately 2-fold in the CDK4 immuno-precipitates.

The induced expression of p21 in myotubes is not reversed by serum restimulation (17, 18), which is in marked contrast to the expression patterns of the D-type cyclins (Fig. 1). To evaluate whether the myogenesis-induced associations of CDK4 with p21 and p27 are a permanent feature of myocyte differentiation, myotube cultures were restimulated with high mitogen growth media, and lysates from these cultures were subjected to immunoprecipitation-coupled immunoblot analysis. As shown in Fig. 7, the levels of CDK4-associated p21 and p27 in serum-restimulated myotubes remained as high as that in myotubes maintained in differentiation media. These data suggest that the sustained association between CDK4 and p21 or p27 is likely to contribute to the repression of CDK4 Rb kinase activity in myotubes after serum stimulation, despite the reinduction of cyclin D1 expression.

**p21-mediated Inhibition of the CDK4 Rb Kinase Activity.** Cell lysate mixing experiments were performed to test for a myogenesis-induced CDK4 inhibitory activity. Some of the CDK inhibitors are stable to heat treatment, and lysates boiled to inactivate the kinases are useful in mixing experiments to assay for these inhibitory activities (18, 22-25). For these experiments, active CDK4 complex was immunoprecipitated from myoblast lysates and incubated with or without heat-treated cell lysates for 30 min at room temperature prior to the Rb kinase assays (Fig. 8A). The inclusion of heat-treated lysates from myotube and serum-restimulated myotube cultures in the Rb kinase reaction markedly reduced the activity of the CDK4 complex, but heat-treated myoblast lysates had little or no effect on CDK4 kinase activity.

### Immunoprecipitation

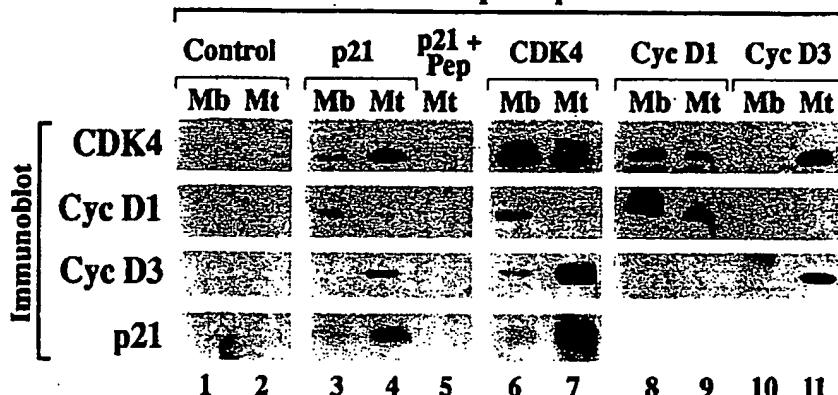


Fig. 5. Immunoprecipitation coupled with immunoblotting analyses of CDK4-associated proteins. Cell lysates prepared from myoblasts (Mb) and myotubes (Mt) were immunoprecipitated with antibodies to the unrelated protein Gax (control, Lanes 1 and 2), anti-p21 (Lanes 3-5), anti-CDK4 (Lanes 6 and 7), anti-cyclin D1 (Lanes 8 and 9), and anti-cyclin D3 (Lanes 10 and 11). The immunoprecipitated protein complexes were separated on SDS-PAGE gels and subjected to immunoblot analyses with CDK4, cyclin D1, cyclin D3, and p21 antibodies (*immunoblot*). In Lane 5, the anti-p21 was preincubated with its immunogenic peptide prior to immunoprecipitation. Anti-p21 immunoblot analysis of cyclin D1 and cyclin D3 immunoprecipitates is not presented, because the immunoglobulin proteins migrate at the same position in this assay.

### Immunoprecipitation

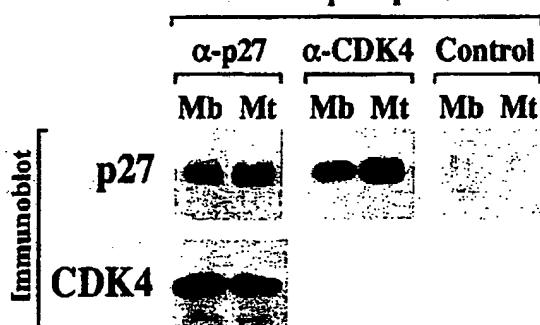


Fig. 6. CDK4 and p27 interactions in C2C12 myocytes. Anti-p27 immunoprecipitates from myoblast (Mb) and myotube (Mt) lysates were subjected to immunoblot analysis with p27 and CDK4 antibodies. Anti-CDK4 immunoprecipitates and immunoprecipitates with a control antibody were also probed with p27 antibody.

To determine whether p21 contributes to the heat-stable CDK4 inhibitory activity that is induced upon myogenesis, heat-treated lysates were first immunodepleted with antibodies raised against peptides specific to p21 and then tested for their CDK4 inhibitory activity (Fig. 8B). The treatment of the myotube lysates with anti-p21 antibodies either partially or completely depleted the CDK4 inhibitory activity that was present in the myotube lysates. The specificity of the p21 immunodepletion was demonstrated by the inclusion of excess p21 immunogenic peptide, which prevented the removal of the CDK4 inhibitory activity. Furthermore, the inclusion of the p21 immunogenic peptide alone had no effect on the CDK4 kinase activity, indicating that it does not interfere with the kinase assay. These data indicate that p21 is a potential inhibitor of the CDK4 activity in myotube lysates.

The analyses represented in Fig. 8, A and B, demonstrate that the p21 inhibitory activity increases upon myogenesis; however, these analyses cannot discern between either the

### CDK4 Immunoprecipitation

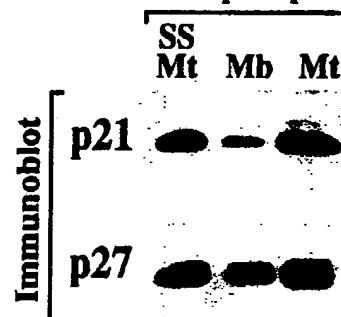
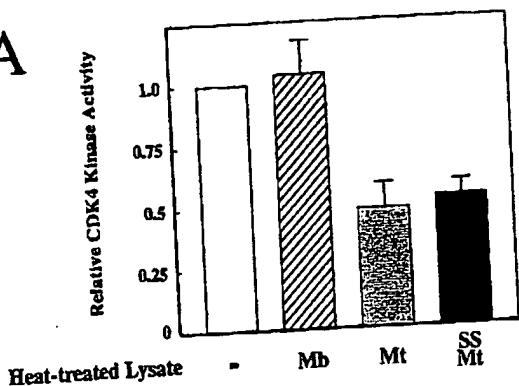


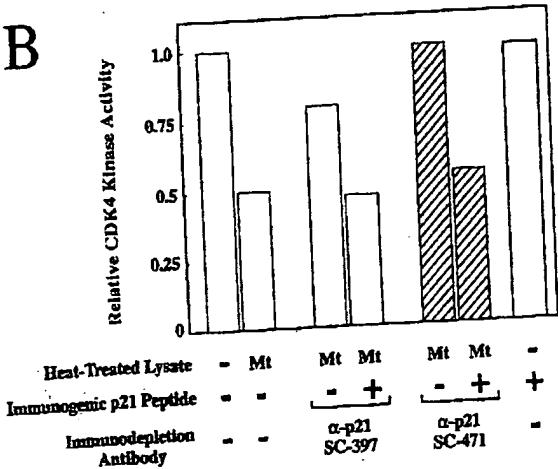
Fig. 7. Sustained association of CDK4 with p21 and p27 in myotubes restimulated with serum. CDK4-associated proteins were immunoprecipitated from lysates of myotubes restimulated with serum (SS), myoblasts (Mb), and myotubes (Mt). The immunoprecipitates were separated on gel and immunoblotted with anti-p21 and anti-p27 antibodies.

possibility that the kinase inhibitory activity is actually bound to CDK4 in myotubes or the possibility that this activity is released from latent, non-CDK4 pools by the heat treatment. Thus, to test whether the myogenesis-induced Rb kinase inhibitory activity is bound to CDK4 in myotubes, the CDK4 protein complexes were immunoprecipitated from myoblast or myotube lysates, boiled in lysis buffer, and then analyzed for their ability to inhibit CDK4 activity. As shown in Fig. 8C, a heat-stable inhibitory activity was detected in CDK4 immunoprecipitates prepared from myotube lysates, but little if any was detected in myoblast lysates. Detection of this inhibitory activity in myotubes was abolished by preincubation with the CDK4 immunogenic peptide prior to immunoprecipitation. This CDK4-bound inhibitory activity was also removed by immunodepleting the myotube lysate with anti-p21 antibody prior to the CDK4 immunoprecipitation step.

A



B



C

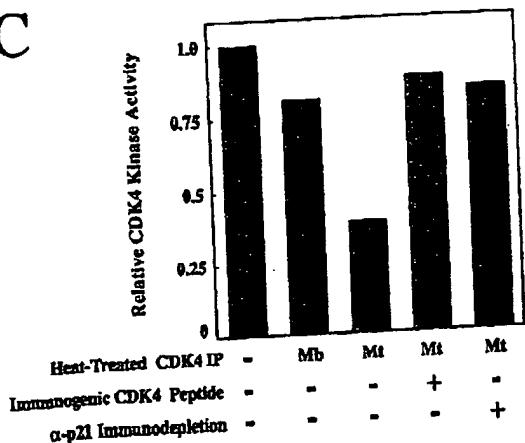


Fig. 8. A, CDK4 inhibitory activity is present in lysates prepared from myotubes and myotubes restimulated with serum. Active CDK4 complexes immunoprecipitated from myoblast lysate were incubated with heat-treated lysates from myoblasts (Mb), myotubes (Mt), myotubes stimulated with serum (SS Mt), or lysate buffer alone (-). The Rb kinase activity of these extracts was then determined as described in "Materials and Methods." The mean values from three independent experiments were plotted as relative kinase activities in the histogram; bars, SE. B, Antibody depletion assays reveal that p21 is a component of CDK4 inhibitory activity in myotube lysates. Heat-treated myotube lysates were incubated with different anti-p21 antibodies (SC-397 and SC-471 from Santa Cruz Biotechnology Inc.) in the presence or absence of their corresponding immunogenic peptides. The p21 immunocomplex was removed with protein A beads by centrifugation. The resulting cleared lysates were used in the CDK4 inhibition assay. As a control, p21 immunogenic peptide

These data demonstrate that p21 is a significant component of the Rb kinase inhibitory activity that is bound to the CDK4 complex in myotubes.

### Discussion

During myogenesis, the expression of CDK4, a major Rb kinase, does not change at the levels of mRNA and protein (Fig. 1; Refs. 14–16). However, the Rb kinase activities of CDK4, cyclin D1, or cyclin D3 immunoprecipitates are reduced significantly. Here, we investigated whether this reduction in Rb kinase activity could result from the differential interactions of CDK4 with CDK inhibitor and cyclin regulatory subunits upon myocyte differentiation.

The reduction in the CDK4 Rb kinase activity upon myogenesis correlated with changes in the subunit composition of CDK4 complex. Immunoprecipitation analyses of <sup>35</sup>S-labeled myocyte lysates revealed four low molecular weight proteins, with apparent molecular weights of  $M_r$  16,000, 21,000, 26,000, and 27,000, which were specifically associated with the CDK4 complex (Fig. 4). Of these, the  $M_r$  21,000, 26,000, and 27,000 protein bands were of greater intensity in the myotube lysates compared with the myoblast lysates. Some cyclin kinase inhibitors are reported to have molecular weights in this range, and immunoprecipitation-coupled immunoblot experiments confirmed that p21 and p27 were associated with CDK4 in the myocyte lysates (Figs. 5 and 6). There was a small but reproducible increase in the interaction between p27 and CDK4 during myogenesis (approximately 2-fold), but the association between CDK4 and p21 increased more substantially (10-fold). In proliferating myoblasts, the low level of p21 (or p27) interaction with CDK4 may function to promote the association between the cyclins and CDK4, leading to the activation of the Rb kinase activity. A similar role for p21 has been described previously for the CDK2 complex (26). However, the large up-regulation of p21 during myogenesis will increase the stoichiometry of the p21 subunit within the CDK4 complex, leading to its inactivation. This hypothesis is supported by the demonstration that anti-p21 antibodies can largely immunodeplete the heat-stable CDK4 and CDK2 inhibitory activities that are present in myotube, but not myoblast, cell lysates (Fig. 8 A and B; Ref. 18). Furthermore, heat-inactivated CDK4 immunoprecipitates from myotubes possess a cyclin kinase inhibitory activity that can be immunodepleted by anti-p21 antibodies (Fig. 8C). Collectively, these data support the hypothesis that the induction of p21 functions to inhibit the Rb kinase activity of

(SC-397P) alone has no significant effect on CDK4 kinase activity. C, p21 is a component of CDK4-bound kinase inhibitory activity in myotubes. CDK4-associated proteins were immunoprecipitated from either myoblast (Mb) or myotube (Mt) lysates. These CDK4 immunoprecipitates were boiled in lysate buffer, and the supernatants were added to active CDK4 complex to test their ability to inhibit the Rb kinase activity. As a control, CDK4 immunogenic peptide was incubated with the CDK4 antibody prior to CDK4 immunoprecipitation. To measure the contribution of p21, myotube lysates were precleared by immunoprecipitation with anti-p21 antibody before performing CDK4 immunoprecipitation. These experiments were repeated twice with different preparation of cell lysates, and similar results were obtained.

CDK4 upon myogenesis, which can lead to the accumulation of hypophosphorylated Rb in myotubes.

The experiments described herein also demonstrate that cyclin D1 is a predominant CDK4-associated cyclin in myoblast lysates, but the level of the cyclin D1-CDK4 complex declines upon myogenesis. Although the levels of cyclin D3 and cyclin D3/CDK4 complex increase during myogenesis, the total level of cyclin D3 in the myotube is not sufficient to form complexes with the majority of CDK4 molecules, and this situation is likely also to contribute to the decrease in CDK4 activity. The immunoprecipitation-coupled immunoblot analyses also did not detect cyclin D1 in cyclin D3 immunoprecipitates nor cyclin D3 in cyclin D1 immunoprecipitates, consistent with the notion that the CDK4 interactions with cyclin D1 and cyclin D3 are mutually exclusive. The cyclin D3-CDK4 complex can phosphorylate Rb *in vitro* (9), and thus, the decline in Rb kinase activity of the cyclin D3 immunoprecipitate upon myogenesis is likely due to the enhanced association of p21 with these complexes. It is paradoxical that cyclin D3 is induced upon myogenesis to become associated with an inactive CDK4 complex, and the significance of cyclin D3 induction during differentiation is not understood at present. One possibility is that cyclin D3, in association with another cyclin kinase partner, may function to phosphorylate myotube-specific proteins. Support for this speculation includes the findings that neurofilament proteins and tau are phosphorylated by CDK5 in postmitotic neurons, demonstrating that this class of kinases can also have differentiation-specific functions (27, 28). It has also been proposed that cyclin D3 functions to sequester p107 in myotubes, which serves to enhance the interaction between E2F and p130 (29).

The data presented here also demonstrate that the myogenesis-induced down-regulation of cyclin D1 and up-regulation of cyclin D3 are reversible in that the levels of these cyclins can be restored partially to their myoblast levels by restimulating cultures of postmitotic myotubes with serum. The reversibility of cyclin D1 and cdc2 mRNA (20) and of cyclin A and CDK2 protein (18) has also been noted in cultures of postmitotic myotubes. In contrast, the up-regulation of p21 during myogenesis is not reversed by serum restimulation (17, 18), and p21 remains bound to the CDK4 complex under these conditions (Fig. 7). Thus, the sustained interaction of p21 with CDK4 may be an important feature that contributes to the irreversibility of cell cycle withdrawal that is exhibited by skeletal muscle cells. Of course, this does not rule out the possibility of other regulatory mechanisms that may also contribute to irreversible CDK4 inactivation and cell cycle arrest. For example, tyrosine phosphorylation of CDK4 has been shown to be required for the G<sub>1</sub> arrest in response to UV irradiation (30), but a potential role for CDK phosphorylation upon myogenesis has yet to be explored.

In summary, the dephosphorylation of Rb is a critical event in myocyte terminal differentiation, yet the protein levels of the Rb kinase do not change upon myogenesis. Here, we demonstrated a marked reduction in the Rb kinase activity of CDK4 that correlates with changes in the proteins that are associated with the CDK4 complex in cell lysates. The decline in CDK4 activity appears to result from a decline in the combined levels of the D-type cyclins and from an irreversible increase in the stoichiometry of the p21 subunit. The

decrease in CDK4 activity may be a mechanism that leads to a reduction in Rb phosphorylation, leading to cell cycle withdrawal upon skeletal muscle differentiation.

## Materials and Methods

**Cell Culture.** Mouse myoblast C2C12 cells were cultured in DMEM supplemented with 20% fetal bovine serum (growth medium). Myogenic differentiation was initiated by shifting subconfluent culture of C2C12 cells to differentiation medium (DMEM and 2% heat-inactivated horse serum). To prepare myotube cultures, myoblasts were cultured in differentiation medium for 2 days, then exposed to 10  $\mu$ M cytosine  $\beta$ -D-arabinofuranoside for 48 h to eliminate undifferentiated myoblasts, and then switched back to differentiation medium for 2 more days. Treated under these conditions, the resulting myotube cultures typically displayed >90% of the nuclei to be present in multinucleated cells by visual examination. Serum restimulation of myotubes was performed by transferring myotube cultures to growth medium for 24 h.

**Antibodies.** Anti-cyclin D1 and anti-cyclin D3 monoclonal antibodies, anti-CDK4 COOH-terminus polyclonal antibody and corresponding immunogenic peptide, anti-p21 and anti-p27 (Kip1) COOH-terminus antibodies, and corresponding immunogenic peptides were all from Santa Cruz Biotechnology. Monoclonal anti-PCNA antibody was also from Santa Cruz Biotechnology. Anti-CDK6 antibody and corresponding immunogenic peptide were a gift from M. Meyerson (Massachusetts General Hospital Cancer Center, Boston, MA) (10). Anti-Rb monoclonal antibody was from PharMingen.

**Immunoblotting.** Cells were lysed in NP40 lysis buffer [0.5% NP40, 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 2  $\mu$ g/ml each of leupeptin and aprotinin], and insoluble materials were cleared off by centrifugation at 12,000  $\times$  g for 10 min. Cell lysates (40  $\mu$ g) were separated on denaturing acrylamide gel and transferred to Immobilon-P membrane (Millipore). The membranes were blocked in 5% milk in PBS with 0.2% Tween 20 and incubated sequentially with the primary and secondary antibodies. Dilution of the primary antibodies was 1:100, as suggested by the manufacturer. The proteins were visualized by using the Amersham enhanced chemiluminescence reagent.

To determine the relative levels of D-type cyclins, CDK4, and p21 in myotube lysates, we devised a semiquantitative immunoblot assay. In these experiments, a series dilution of recombinant GST fusions of CDK4, cyclin D1, cyclin D3, and p21 (Santa Cruz Biotechnology, Inc.), ranging from 1 to 100 ng, and 40  $\mu$ g of myotube lysate were electrophoresed on the same gel and subjected to immunoblotting. Estimates of protein levels in cell lysates were obtained by comparing the immunoblot band intensity from cell lysates with that of known amounts of recombinant protein that were loaded onto adjacent lanes in the denaturing acrylamide gel. Band intensities were quantified on an Eagle-Eye densitometer (Stratagene). To avoid possible variation of results due to antibody availability, the experiments were repeated several times with different dilutions of antibodies (1–4  $\mu$ g/ml), and similar results were obtained. Levels of the cell cycle proteins, expressed as fmol/ $\mu$ g of myotube lysate, were calculated using the molecular weights of GST fusion proteins.

**Metabolic Labeling and Immunoprecipitation.** Cells were labeled by incubating in DMEM, without methionine and cysteine, supplemented with dialyzed fetal bovine serum and 100  $\mu$ Ci/ml each of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 4 h at 37°C. Cell lysates were prepared as described above. The cell lysates were precleared once by incubation with nonspecific serum and protein A beads (Santa Cruz Biotechnology, Inc.). For immunoprecipitation, cleared lysates were incubated with 1  $\mu$ g of the specific antibodies and protein A beads for 3 h at 4°C. Protein complexes collected on the beads were washed five times with lysis buffer and separated on 12% SDS-PAGE gel. The <sup>35</sup>S-labeled protein bands were visualized by fluorography. For peptide competition, the antibodies were first incubated with 2  $\mu$ g of corresponding immunogenic peptides for 15 min at room temperature before performing the immunoprecipitation. For immunoprecipitation-coupled immunoblotting, immunoprecipitated protein complexes from 500  $\mu$ g of cell lysates were eluted from the protein A beads by boiling in 1 $\times$  SDS sampling buffer (as in Ref. 3) and subjected to immunoblotting analysis.

**In Vitro Rb Kinase and CDK4 Inhibition Assay.** *In vitro* Rb kinase assay was performed as described (9) by using as substrate a protein

covering the COOH terminus (605-821) of mouse Rb protein that was fused to GST (GST-Rb; Ref. 3). Briefly, cyclin kinase complexes were immunoprecipitated from cell lysates (200 µg) with the specific antibodies and collected on protein A beads. The beads were washed twice with kinase buffer [50 mM Tris (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of leupeptin and aprotinin] and incubated with 2 µg of GST-Rb fusion protein and 4 µCi [ $\gamma$ -<sup>32</sup>P]ATP in 50 µl of kinase buffer for 30 min at room temperature. Reactions were terminated by the addition of 20 µl of 4X SDS sample buffer and boiled for 5 min. Samples were separated by electrophoresis on polyacrylamide gel, and the phosphorylated proteins were visualized by autoradiography of the dried gels. For quantitative measurement of the CDK4 kinase activities, the intensity of each band on autoradiography films were scanned with an Eagle-Eye densitometer (Stratagene) and expressed in the histogram as relative kinase activity to that of CDK4 complex from myoblast lysates. The CDK2-associated histone H1 kinase activity was measured as described (18).

The GST-Rb fusion protein was purified from *Escherichia coli* extracts by batch chromatography using glutathione-Sepharose 4B beads (Pharmacia). GST-Rb was eluted from the beads by incubation in kinase buffer with 5 mM reduced glutathione at 4°C. Eluted proteins were visualized by staining with Coomassie blue following electrophoresis on a denaturing polyacrylamide gel. The concentration of GST-Rb fusion proteins was estimated by comparing the density of GST-Rb band to that of protein standards of known mass.

For CDK4 inhibition assays, cell lysates were boiled for 5 min to release the bound CDK inhibitors and cleared by brief centrifugation in an Eppendorf tube. These heat-treated cell lysates were then incubated with active CDK4 complexes immunoprecipitated from myoblast lysates for 30 min at room temperature. The protein complexes were washed with kinase buffer, and Rb kinase assay was performed as described above. For analysis of CDK inhibitory activity in CDK4 immunoprecipitates, cell lysates were incubated with anti-CDK4 antibody and protein A beads for 2 h at 4°C and washed three times with lysis buffer. The beads were boiled for 5 min in 0.5 ml of lysis buffer and centrifuged. The supernatants were collected and mixed with active CDK4 proteins complexes to assay their inhibitory activities as described above. For peptide competition, immunogenic CDK4 peptide (1 µg) was included in the CDK4 immunoprecipitation reaction. For p21 depletion, cell lysates were first immunoprecipitated with anti-p21 antibody prior to CDK4 immunoprecipitation.

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# Involvement of p27<sup>Kip1</sup> in G1 arrest by high dose 5 $\alpha$ -dihydrotestosterone in LNCaP human prostate cancer cells

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The cell cycle is governed by cyclin dependent kinases (cdks), which are activated by binding of cyclins, inhibited by cdk inhibitors and regulated by phosphorylation and dephosphorylation. Exposure to high dose dihydrotestosterone (DHT) inhibits population growth of the human prostate carcinoma cell line, LNCaP. To determine the mechanism of growth arrest by high dose DHT, we assayed the changes in cell cycle profile and the cell cycle regulators that mediate these effects. Treatment of asynchronously growing LNCaP cells with 100 nM DHT caused a G1 arrest. The proportion of cells in S phase fell from 22 to 2%, while the G1 fraction rose from 74 to 92% by 24 h. Loss of phosphorylation of the retinoblastoma protein was noted and cdk4 and cyclin E/cdk2 activities fell. Inhibition of these G1 cyclin dependent kinases was not due to loss of either cyclin or cdk proteins nor to increases in the cdk inhibitors p16<sup>INK4a</sup> and p21<sup>Cip1</sup>. p21<sup>Cip1</sup> protein levels remained constant, and cyclin E-associated p21<sup>Cip1</sup> fell, suggesting that p21<sup>Cip1</sup> is not relevant to this form of cyclin E/cdk2 inhibition. Of note, total p27<sup>Kip1</sup> levels and cyclin E-associated p27<sup>Kip1</sup> increased as cells arrested and the amount of the CAK activated cdk2 bound to cyclin E decreased. p27<sup>Kip1</sup> immunodepletion experiments demonstrated that the DHT-mediated increase in p27<sup>Kip1</sup> was sufficient to fully saturate and inhibit target cyclin E/cdk2. The inhibition of cyclin E/cdk2 by p27<sup>Kip1</sup> contributes to G1 arrest of LNCaP following high dose DHT. p27<sup>Kip1</sup> may be a key effector of androgen dependent growth modulation in prostate cancer cells. *Oncogene* (2000) 19, 670-679.

**Keywords:** p27; cell cycle; prostate cancer; androgen; cyclin E/cdk2

## Introduction

Androgen interaction with the androgen receptor (AR) is important for growth and development of both the normal prostate and of prostate cancer (Hakimi *et al.*, 1996). At present, the most effective therapy for prostate cancer is reduction of testosterone or of its most active metabolite, 5 $\alpha$ -dihydrotestosterone (DHT)

by different treatments collectively referred to as androgen ablation (Catalona, 1994). However, tumor progression to an androgen insensitive state severely limits the efficacy of these treatments. We have investigated how this steroid pathway influences key cell cycle regulators in the androgen sensitive, human prostate cancer cell line, LNCaP. An understanding of how cell cycle progression is influenced by androgen pathways, and how these mechanisms are disrupted in prostate cancer progression, may lead ultimately to new methods of achieving cytostasis in hormone resistant prostate cancers.

Progression through the cell cycle is governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by phosphorylation (Solomon, 1993), activated by binding of cyclins (Morgan, 1995; Sherr, 1994) and inhibited by the cdk inhibitors (Reed *et al.*, 1994; Sherr and Roberts, 1995). The cdk5s regulate biochemical pathways, or checkpoints, which integrate mitogenic and growth inhibitory signals, monitor chromosome integrity, and coordinate cell cycle transitions (Hartwell, 1992; Murray, 1992). Passage through G1 into S phase is regulated by the activities of cyclin D-, cyclin E-, and cyclin A-associated kinases. Cyclin B-dependent kinases regulate the G2/M transition.

Two families of cdk inhibitors mediate cell cycle arrest following growth inhibitory stimuli (Sherr and Roberts, 1995, 1999). The inhibitor of cdk4, INK4, family members p15<sup>INK4b</sup>, p16<sup>INK4a</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup> bind cdk4 and cdk6 specifically and inhibit cyclin D binding. Members of the KIP or kinase inhibitor protein family, which include p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>kip2</sup>, bind and inhibit target cyclin/cdk complexes. The cdk inhibitor, p27, acts during G0 and the early G1 phase of the cell cycle to inhibit G1 cyclin/cdk complexes (Polyak *et al.*, 1994a,b; Toyoshima and Hunter, 1994; Slingerland *et al.*, 1994; Hengst *et al.*, 1994). In many cell types, p27 is essential for quiescence. Antisense p27 inhibits exit from the cell cycle following serum withdrawal (Coats *et al.*, 1996; Rivard *et al.*, 1996) and antisense p27 stimulates estradiol depleted quiescent MCF-7 breast cancer cells to re-enter cell cycle (Cariou, Donovan and Slingerland, in preparation). p27 levels are regulated by post-transcriptional mechanisms including both translation and proteolysis (Hengst and Reed, 1996; Pagano *et al.*, 1995; Millard *et al.*, 1997). In human cell lines, phosphorylation of p27 is maximal in G1, just prior to the drop in p27 protein that occurs at S phase (Pagano *et al.*, 1996). Phosphorylation of p27 triggers its proteolytic degradation (Vlach *et al.*, 1997; Sheaff *et al.*,

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al., 1997; Montagnoli *et al.*, 1999; Tsvetkov *et al.*, 1999), as is the case for the yeast cdk inhibitor Sic1 (Schneider *et al.*, 1996; Tyers, 1996).

p27 increases during differentiation in many cell types, including differentiation induced by vitamin D3 (Hengst and Reed, 1996; Wang *et al.*, 1996) and by androgen in the prostatic epithelium (Chen *et al.*, 1996). p27 knockout cells manifest altered differentiation programs (Casaccia-Bonelli *et al.*, 1997). Indeed, p27 knockout mice develop multiple organ hyperplasia (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996), including prostatic hyperplasia (Cordon-Cardo *et al.*, 1998), underlining the importance of p27 as an inhibitor of prostate cell proliferation.

Increasing evidence suggests that the cyclins, cdk's and cdk inhibitors are either themselves targets for genetic change in cancer or are disrupted secondarily by other oncogenic events (Hunter and Pines, 1994). Since they oppose mitogenic stimuli, the cdk inhibitors are good candidates as tumor suppressors. Although genetic changes in the *p16* gene support a tumor suppressor (TS) role for this inhibitor in cancers (Bates and Peters, 1995), mutations in *p27* have been identified only rarely (Kawamata *et al.*, 1995; Pietenpol *et al.*, 1995; Ponce-Castaneda *et al.*, 1995). A reduction in p27 protein could contribute to resistance to growth inhibitory factors, deregulation of cell proliferation, and oncogenic change. Although normal human epithelia of the breast, prostate, lung, and colon express high levels of p27 protein, this protein is frequently reduced in primary carcinomas at these sites (reviewed in Cariou *et al.*, 1998). The reduction of p27 protein is of prognostic importance in these cancers and correlates with an aggressive tumor phenotype *in vivo* (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Porter *et al.*, 1997; Tan *et al.*, 1997; Mori *et al.*, 1997; Esposito *et al.*, 1997; Tsihlias *et al.*, 1998). Enhanced proteolytic degradation may underlie the loss of p27 in tumor cells (Catzavelos *et al.*, 1997; Loda *et al.*, 1997; Esposito *et al.*, 1997). Thus, changes in the post-translational mechanisms which target p27 for degradation, may be germane to oncogenesis and/or tumor progression.

In a study of p27 immunostaining in primary prostate cancer, we found that loss of p27 protein was an independent prognostic factor, predicting reduced time to recurrence following radical prostatectomy ( $P=0.047$ , risk ratio 2.08) (Tsihlias *et al.*, 1998). In a small subset of patients, androgen ablation therapy prior to tumor removal appeared to increase p27 protein, raising the possibility that androgens may modulate p27 degradation pathways *in vivo*. In the present study, the effect of high dose androgen on cell cycle progression was investigated in a model of human prostate cancer, the LNCaP cell line.

LNCaP is an androgen-sensitive human prostate cancer cell line derived from a metastasis to a supraclavicular lymph node (Horoszewicz *et al.*, 1983). The line has an aneuploid human male karyotype, and is tumorigenic when implanted into nude mice. The potent androgen, 5 $\alpha$ -dihydrotestosterone (DHT), modulates LNCaP proliferation in a well-characterized fashion. DHT stimulates proliferation at concentrations between 0.1 and 1.0 nM DHT and proliferation is inhibited at DHT concentrations below 0.1 nM or above 10 nM DHT. Androgens have also been shown to mediate biphasic growth response in the prostate *in vivo*. Low

dose androgen administered to castrate rats will reverse prostate atrophy and induce proliferation in the prostate gland. Higher doses of androgen cause growth arrest by inducing differentiation of prostate epithelial cells (Chen *et al.*, 1996). Because our study of primary tumors suggested that p27 regulation may be importantly altered in human prostate cancers, we wished to explore further how androgen may modulate the cell cycle and p27 function in the prostate cancer line, LNCaP.

## Results

### 100 nM dihydrotestosterone causes G1 arrest of LNCaP

Treatment of asynchronously growing LNCaP cells with 100 nM DHT for 24 h induced a G1 arrest. The proportion of cells in G1 went from 70% in the asynchronously growing untreated cells, to 92% at

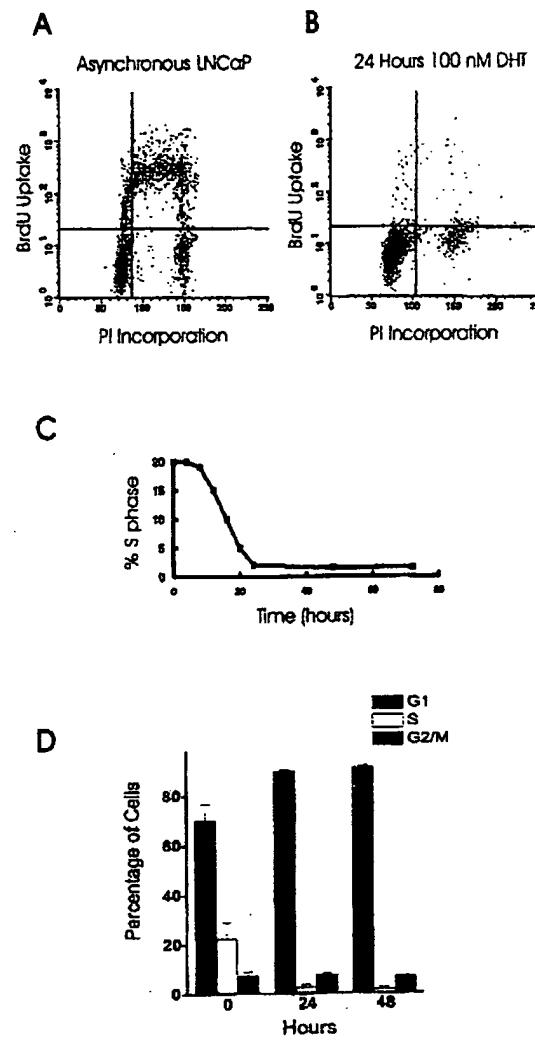


Figure 1 FACS analysis of LNCaP treated with 100 nM dihydrotestosterone. (a) Dot plot showing relative uptake of BrdU and PI in asynchronously growing LNCaP. (b) Decreased BrdU uptake indicating arrest of DNA synthesis after 24 h treatment with 100 nM DHT. (c and d) Cells were harvested and prepared for FACS analysis at various intervals after addition of 100 nM DHT to culture media. G1 arrest was maximal at 24 h. Results shown in (d) are the average of three separate experiments

maximal arrest by 48 h. Cells in S phase fell from 22% of the untreated population to 2% within 24 h (Figure 1). A sub-G1 population was not observed. No significant growth arrest was observed when cells were cultured in media supplemented with 1 and 10 nM DHT (data not shown).

The cell cycle arrest persisted for up to 96 h in media supplemented with DHT. This DHT-mediated cell cycle arrest was not accompanied by cytotoxicity, based on morphological appearance and the use of Trypan blue exclusion staining. The proportion of cells that failed to exclude Trypan blue was similar in treated and untreated cells. Treatment of LNCaP cells with 50 nM DHT also caused a G1 arrest within 48 h. To determine whether high dose DHT was inducing a form of irreversible differentiation, cells were cultured for 48 h in 50 and 100 nM DHT and then transferred to media without additional DHT for a further 24 h. Upon removal of supplemental DHT from the culture media, cells underwent a prompt release from G1 arrest, showing an asynchronous cell cycle profile within 24 h (S phase fraction 20%) (Table 1).

To determine whether the effects of 100 nM DHT on prostate cancer cell proliferation could occur in the absence of the AR and AR signaling, two steroid-independent prostate cancer lines, PC-3 (Kaighn *et al.*, 1979) and DU145 (Stone *et al.*, 1978) were treated with 100 nM DHT. Neither the PC-3 nor the DU145 prostate cancer cell lines express detectable AR (Kaighn *et al.*, 1979; Stone *et al.*, 1978). The addition of 100 nM DHT to the culture medium had no effect on either PC-3 or DU145 cell proliferation (data not shown). Treatment of LNCaP cells with 100 nM DHT for 48 h led to an increased secretion of prostate specific antigen as determined by Hybritech assay on culture media. PSA concentration (ng/ml) in conditioned media from cells with/without 100 nM DHT was 383.5/167.5 and 443.1/340.3 on two separate occasions. Although the results were not normalized for cell number, cells were initially plated equally. After 48 h, the number of cells on DHT treated plates would be less than that on untreated plates. Thus, relative cellular production of secreted PSA was increased by exposure to 100 nM DHT. A Western blot did not demonstrate any difference in cellular PSA levels as cells underwent arrest (data not shown).

*DHT mediated G1 arrest is accompanied by accumulation of nuclear p27 protein*

Immunohistochemistry performed on asynchronous populations of LNCaP cells demonstrated a heterogeneous nuclear immunoreactivity for p27. Cells

Culture conditions	%G1	%S	%G2/M
Asynchronously growing cells	76	19	5
100 nM DHT 48 h	93	2	5
100 nM DHT 48 h then no DHT 24 h	74	19	7
50 nM DHT 48 h	93	2	5
50 nM DHT 48 h then no DHT 24 h	72	20	8

Asynchronously growing cells were cultured in RPMI 1640 plus 5% FBS with either 50 or 100 nM 5 $\alpha$ -dihydrotestosterone (DHT) for 48 h. Cells were then either collected for FACS analysis or washed and fresh media without supplemental DHT was added to plates for a further 24 h prior to FACS analysis

arrested in G1 after treatment with 100 nM DHT for 48 h demonstrated uniformly strong nuclear staining for p27 protein (Figure 2). Controls reacted with secondary antibody alone did not show any significant nuclear staining. Pre-adsorption of the primary antibody with blocking peptide abolished the nuclear staining (data not shown).

Western analysis at intervals after treatment with 100 nM DHT showed loss of phosphorylation of the retinoblastoma protein (pRb) within 16 h, with predominantly hypophosphorylated pRb detected at 48 h. There was no appreciable change in the protein levels of cdk2, cdk4, and cdk6, cyclins D1 and E, or the cdk inhibitors p16<sup>INK4a</sup> and p21<sup>Cip1</sup>. Levels of cyclins A and B1 fell significantly. This is consistent with the DHT-mediated inhibition of entrance into S and G2/M phases, where peak expression of these latter two proteins is known to occur. An increase in p27 protein levels was appreciable within 4 h after addition of DHT, with peak levels fourfold above baseline detected by 48 h, as determined by densitometry (Figure 3).

*The increase in p27 saturates and inhibits cyclin E/cdk2 in DHT-arrested LNCaP*

Inhibition of cyclin E/cdk2 activity was notable within 12 h after the addition of 100 nM DHT to the asynchronously growing cells, with minimal activity detected at 24 h (Figure 4b). To further investigate the mechanism of inhibition of cyclin E/cdk2, these complexes were examined by immunoprecipitation of cyclin E followed by Western blotting to detect associated proteins. No dissociation of cyclin E/cdk2 complexes was observed, however, the amount of CAK-activated threonine 160 phosphorylated cdk2 bound to cyclin E decreased. Phosphorylation by cdk activating kinase (CAK) shifts cdk2 to its faster mobility form on SDS-PAGE (Gu *et al.*, 1992). The amount of cyclin E-associated p21 fell, suggesting that p21 is not relevant to this form of cyclin E/cdk2 inhibition. The amount of cyclin E-associated p27 increased 3–4-fold, by densitometry, as the cells entered G1 arrest (Figure 4a). In other cellular contexts, such an increase in p27 has been shown to saturate cellular cyclin E/cdk2 (Reynisdottir *et al.*, 1995).

To determine whether this increase in p27 was sufficient to saturate target cyclin E/cdk2 and induce cell cycle arrest, p27 was immunodepleted by three serial immunoprecipitations from both asynchronously growing cell lysates and from DHT-arrested cell lysates. Cyclin E immune complexes were examined before and after p27-immunodepletion. p27 immunodepletion caused a significant reduction of cyclin E protein from asynchronously growing cell lysates (Figure 4c). This reflects the substantial proportion of G1 phase cells in an asynchronous population of LNCaP. In spite of a significant reduction in the level of cyclin E, the remaining cyclin E immune complexes showed almost the same kinase activity after p27 immunodepletion as before (Figure 4c, left panel). This is consistent with most p27-bound cyclin E complexes having minimal kinase activity. There was virtually no detectable cyclin E remaining after p27 immunodepletion of DHT-arrested cell lysates. Thus, the increase in p27 by DHT treatment was sufficient to saturate and inhibit the cellular cyclin E/cdk2 complexes.

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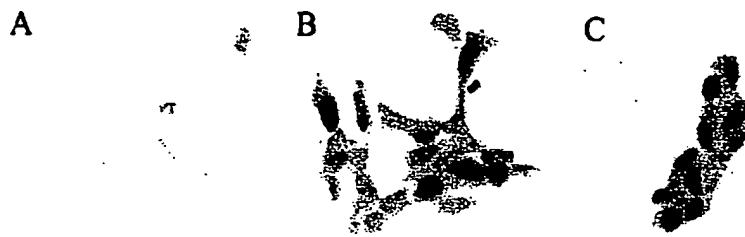


Figure 2 p27 nuclear staining of LNCaP cells in tissue culture. Cells were cultured on poly-L-lysine coated glass slides, fixed and stained for p27 as described in the Materials and methods section. (a) Control sample obtained by staining with secondary antibody alone. (b) Asynchronously growing cells. (c) Cells arrested by treatment with 100 nM DHT show uniform strong nuclear p27 staining

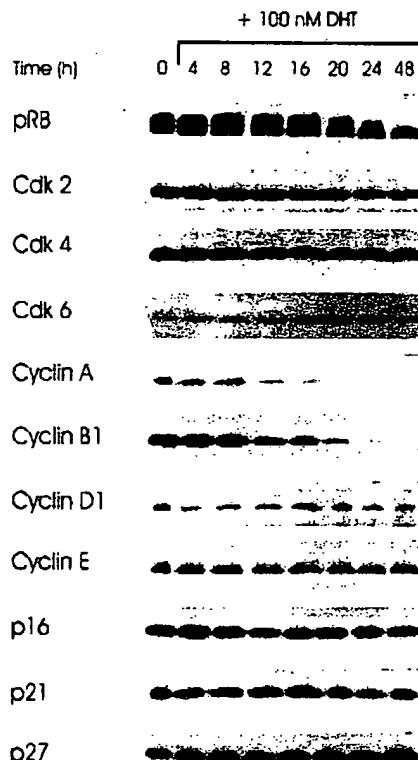


Figure 3 Cell cycle regulators during G1 arrest by DHT. Cells were harvested at indicated time intervals after addition of 100 nM DHT to culture media. Lysates were prepared and resolved using 17.5% SDS-PAGE. Proteins were detected by immunoblotting with the indicated antibodies

#### Antisense p27 failed to prevent the increased binding of p27 to cyclin E after high dose DHT

Antisense p27 oligonucleotides (ASp27) were transfected into asynchronously growing LNCaP cells. Six hours post transfection, 100 nM DHT was added to control (lipid alone), mismatch oligonucleotide (MSp27) and ASp27 transfected cells. In both oligonucleotide-transfected and non-transfected controls, 100 nM DHT induced G1 arrest. MSp27 and control cells showed the same increase in p27 by DHT as did LNCaP treated with 100 nM DHT alone. ASp27 prevented the fourfold rise in p27 protein following DHT treatment. p27 levels in the cells treated with

ASp27 and DHT together were similar to untreated asynchronously growing LNCaP. However, when cyclin E immune complexes were analysed, ASp27 treated cells still showed an increase in the binding of p27 to cyclin E complexes following 100 nM DHT, in spite of the modest reduction in total p27 levels. ASp27 treatment did not reduce total p27 protein levels to a sufficient degree in LNCaP to prevent the DHT-mediated increased association of p27 with its target kinase cyclin E/cdk2 (data not shown).

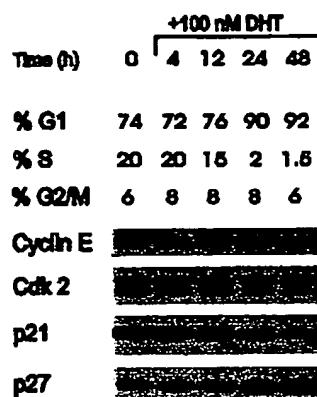
#### Inhibition of cyclin D-bound cdk4 by high dose DHT

The cyclin D-associated kinases also function in G1 to regulate the transition into S phase. To assay the effects of 100 nM DHT on these complexes, cdk4 was immunoprecipitated and kinase activity assayed at various intervals during the arrest of the LNCaP cells. Cdk4 activity was strongly inhibited by treatment with 100 nM DHT (Figure 5b). Immunoprecipitation of cdk4 and its associated complexes did not demonstrate any dissociation of cyclin D1 from cdk4. The level of cdk4-bound p16 remained unchanged. There was a rise in p15 association with cdk4 between 0–12 h after addition of DHT, which persisted to 24 h. Between 24–48 h, there was no further reduction in the per cent S phase cells, and cdk4-bound p15 actually decreased slightly by 48 h, possibly reflecting progression into a quiescent state. The levels of p21 and p27 bound to cdk4 decreased as cells underwent arrest.

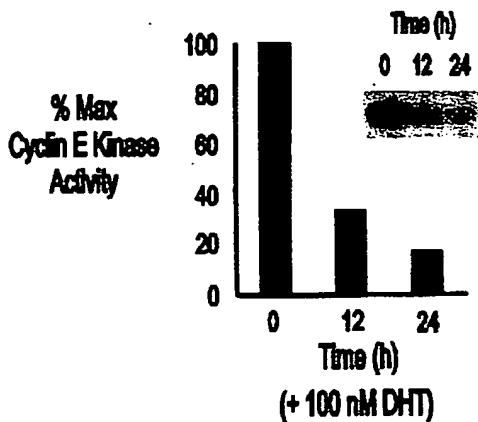
#### Discussion

The LNCaP cell line is the most commonly studied model of androgen-sensitive prostate cancer. This cell line shows a characteristic bell shaped growth curve in response to increasing concentrations of androgen in the growth media (Horoszewicz *et al.*, 1983; Lee *et al.*, 1995; Kim *et al.*, 1996). Although the dose-dependent differences in the effects androgen on cell numbers have been known for nearly two decades, the mechanism of the growth inhibition by high dose androgen was poorly understood. Androgens are required for normal growth and differentiation in prostate epithelial cells. Chen *et al.* (1996) studied the effects of androgen on the cell cycle of normal prostate *in vivo* in rats. Castration resulted in atrophy of the gland due to

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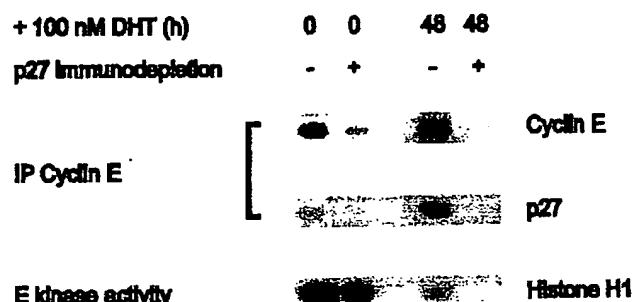


Figure 4 p27 saturates cyclin E/cdk2 complexes and inhibits this kinase following treatment of asynchronously growing LNCaP with 100 nM DHT. (a) Cyclin E immune complexes. Cells were recovered at intervals after addition of DHT to culture media. DNA profiles were determined by FACS analysis, and the percentage of cells in G1, S, and G2/M is indicated for each time point. Cyclin E was immunoprecipitated, complexes resolved by SDS-PAGE and immunoblots were reacted with antibodies to detect associated proteins. (b) Cyclin E/cdk2 Kinase Activity. Cyclin E was immunoprecipitated from lysates collected in (a) above and associated kinase activities were assayed. Reaction products were resolved by SDS-PAGE, and gels were dried and exposed to film. Radioactivity in the Histone H1 substrate was quantitated by PhosphorImager and expressed as a percentage of maximum activity after subtraction of background from control immunoprecipitates. (c) p27 immunodepletion experiments. p27 was serially immunodepleted from lysates collected at either 0 or 48 h after addition of 100 nM DHT to media. Cyclin E-associated proteins and cyclin E-associated kinase activities were assayed before and after p27 immunodepletion from the T=0 and T=48 h samples

apoptosis and arrest of surviving cells. Re-administration of testosterone induced transient epithelial cell proliferation followed by decreased cellular proliferation and an increase in p27. It was postulated that the increased p27 was playing a role to inhibit proliferation as regenerating prostatic tissue underwent steroid-mediated differentiation. The paradoxical effects of androgen stimulation in LNCaP (growth stimulation at low concentrations of androgen, growth inhibition with increased PSA secretion at high concentrations) may represent a normal physiological mechanism that has been retained by this tumor cell line.

Several lines of evidence suggest that the G1 arrest by high dose DHT is an AR mediated effect. In our study, as in others (Lee *et al.*, 1995), high dose DHT induced an increase in PSA secretion by the LNCaP cells. PSA is a serine protease that is normally secreted by the prostatic epithelium. We demonstrated higher PSA production in conditioned media from cells arrested after 48 h in 100 nM DHT than in cells without added androgen. This result and our observation that cellular PSA levels were not increased by

Western analysis are consistent with a DHT-induced increase in secretion of PSA. Treatment of AR-negative PC-3 cells and DU145 cells with 100 nM DHT affected neither cellular proliferation nor PSA secretion (not shown). Furthermore, PC-3 cells that have been genetically engineered to express the AR show growth arrest in response to androgen treatment (E Brown, personal communication).

The present study demonstrates that the decreased rate of growth for LNCaP at high doses of DHT is due to G1 arrest and not to cell death. Growth inhibition with G1 arrest was demonstrated at doses of 50 and 100 nM DHT, whereas, no significant growth arrest was observed with doses between 1–10 nM DHT. We saw no morphological evidence of cell toxicity and Trypan blue exclusion did not differ between treated and untreated cells. Furthermore, the G1 arrest appeared fully reversible on removal of the high dose DHT at 48 h. The longest treatment interval was 96 h, with maximal arrest noted at 24 h. Whether exposure to 100 nM DHT longer than 96 h might induce an irreversible G1 arrest is not known.

## A IP Cdk4 B

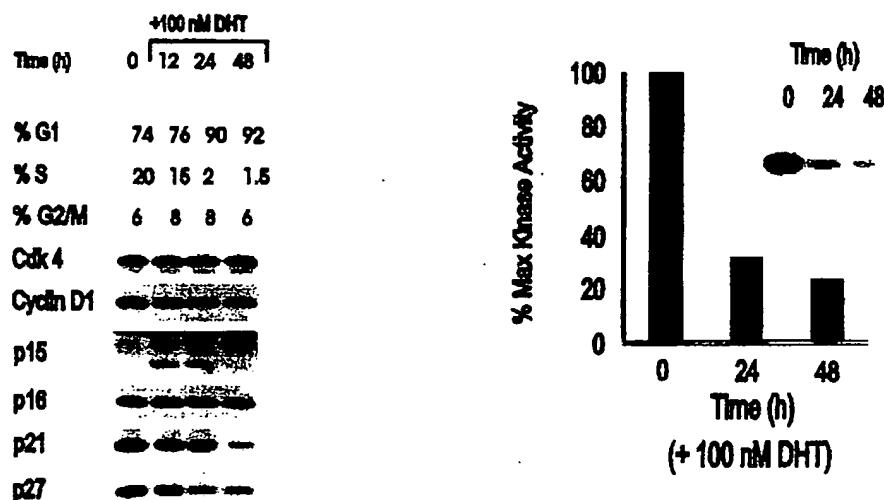


Figure 5 Inhibition of Cdk4 activity during DHT-mediated G<sub>1</sub> arrest. Asynchronous LNCaP cultures were treated with 100 nM DHT and cells were recovered for FACS analysis and preparation of protein lysates. (a) Cdk4 complexes. Cdk4 was immunoprecipitated and complexes were resolved and immunoblotted as for Figure 4a. (b) Cdk4 kinase assay. Cdk4 was immunoprecipitated and kinase activity was assayed as in Materials and methods

High dose DHT caused a loss of pRB phosphorylation as has been observed in other forms of G<sub>1</sub> arrest (Laiho *et al.*, 1990; Sandhu *et al.*, 1997). Hypophosphorylated pRB binds and inactivates E2F, thereby inhibiting the function of that transcription factor, and precluding transcription of genes required for S phase entry (DeGregori *et al.*, 1995). Phosphorylation of pRB leads to its dissociation from E2F, and to E2F activation (Chellappan *et al.*, 1991). The pRB gene is wild type in LNCaP (Peehl, 1994). Since both cyclin D-associated kinases and cyclin E/cdk2 activities contribute to pRB phosphorylation (Sherr and Roberts, 1995), the effects of DHT on these cyclin/cdk's were assayed.

Cyclin E-dependent kinase activity fell as cells underwent arrest. This was accompanied by a fourfold overall increase in p27 protein. More importantly, the increase in p27 protein was sufficient to fully saturate cyclin E/cdk2 complexes. p27-immunodepletion demonstrated that essentially all of the cellular cyclin E was bound to p27 in these DHT-arrested cells. This supports the conclusion that the increase in p27 contributes causally to cyclin E/cdk2 inhibition and to the G<sub>1</sub> arrest observed. In other systems, a similar increase in p27 levels has been shown to be sufficient to saturate and inactivate cyclin E/cdk2 (Reynisdottir *et al.*, 1995). The fact that cyclin E-associated p21 fell as cells arrested, suggests that p21 is not likely to be involved in inhibition of this kinase.

ASp27 treatment prevented the fourfold increase in p27 levels following exposure to 100 nM DHT. However, ASp27 failed to prevent DHT-mediated G<sub>1</sub> arrest. In spite of the modest reduction in p27 protein levels, the increased association of p27 with cyclin E/cdk2 complexes was intact in ASp27 treated cells arrested by 100 nM DHT. This suggests that factors that lead to an increased affinity of binding of p27 to

cyclin E/cdk2 following DHT treatment may be more important to the inhibition of this kinase than the increase in p27 protein levels alone.

Cdk activating kinase (CAK) mediated phosphorylation of cdk2 on threonine 160 is required for catalytic activity (Gu *et al.*, 1992). In this study, cyclin E-bound cdk2 showed a progressive loss of CAK activation, with accumulation of the slower mobility form of cyclin E-bound cdk2. Kato *et al.* (1994) have demonstrated that p27 can inhibit CAK activation of cyclin D-associated cdk4 during cyclic AMP mediated G<sub>1</sub> arrest in macrophages. Increased binding of p27 to cyclin E/cdk2 may modulate the conformation of these complexes and block the access of CAK to its catalytic sites on cdk2, thereby preventing the activating phosphorylation of cdk2. A similar loss of CAK activation of cyclin E-bound cdk2 has been demonstrated in TGF- $\beta$  arrested cells (Koff *et al.*, 1993; Slingerland *et al.*, 1994).

Cdk4 kinase activity was also inhibited by 100 nM DHT. p16, p21, and p27 are not likely involved in the inhibition of this kinase, since p16 association with cdk4 remained constant and dissociation of p21 and p27 from cdk4 was observed. p15 binding to cdk4 was increased at 12 h and remained elevated until 24 h. The accumulation of p15 may facilitate dissociation of p21 and p27 from cdk4 complexes. Versions of this model of action for p15 have been suggested previously in G<sub>1</sub> arrest due to TGF- $\beta$  (Reynisdottir *et al.*, 1995; Sandhu *et al.*, 1997). It is also possible that the affinity of p27 for cyclin E/cdk2 is actively regulated as suggested by Sheaff *et al.* (1997), and this may be independent of the effects of p15 on cyclin D/cdk4 complexes (Sandhu *et al.*, 1997).

The INK4 family of cdk inhibitors have been shown to inactivate their target kinases by binding to them and displacing the associated cyclin (Parry *et al.*, 1995;

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Sandhu *et al.*, 1997). Although we did not see cyclin D1 dissociation from cdk4 as kinase activity diminished, it is possible that p15 may have displaced cyclin D2 or D3. However, we were unable to detect cyclins D2 or D3 in cdk4 complexes using commercially available antibodies. Clearly, there may be more than one mechanism contributing to DHT-mediated G1 arrest. Although p27 appears to be important in the inhibition of cyclin E/cdk2, other mechanisms, including p15, may act to inhibit the D-type cyclin/cdk5.

Since 1941, when Huggins and Hodges discovered that prostate cancer undergoes regression in response to castration, there have been few major advances in the management of metastatic prostate cancer (Huggins *et al.*, 1941). Leutinizing hormone releasing hormone (LHRH) analogs that induce castrate levels of testosterone have obviated the need for castration (Peeling, 1989). In spite of advances in the hormonal therapy of prostate cancers, progression to a hormone independent state invariably occurs. Thus, there is clearly a need for development of novel treatment approaches for advanced prostate cancer. A greater understanding of how cell cycle regulators respond to androgenic stimuli in normal and malignant prostatic epithelial cells could lead to the identification of new targets for therapy. The present study suggests that the increased levels of p27, and its binding and inhibition of cyclin E/cdk2 contribute to G1 arrest of LNCaP human prostate cancer cells in response to high doses of DHT.

Several other reports suggest a role for p27 as a mediator of G1 arrest in prostate cancer tissue culture models. G1 arrest of prostate cancer line DU145 induced by EGF receptor blockade involves upregulation of p27 protein and corresponding inhibition of cyclin E/cdk2 activity (Peng *et al.*, 1996; Zi *et al.*, 1998). A subline of LNCaP developed by continuous culture in the absence of androgen is growth inhibited by low doses of androgen. In this LNCaP subline, loss of cdk2 activity was also correlated with accumulation of p27 (Kokontis *et al.*, 1998). In an androgen dependent mouse mammary carcinoma cell line, SC-3, G1 arrest upon androgen withdrawal is associated with a similar upregulation of p27 and the binding and inhibition of cdk2 by p27 (Menjo *et al.*, 1998). In these different tissue culture models, androgen mediates different effects on proliferation: in the one case, androgen induces growth arrest, whereas in the other, androgen withdrawal has this same effect. Interestingly, in each case, a similar mechanism of G1 arrest appears to be invoked, with upregulation of p27 leading to inhibition of cyclin E/cdk2 activity. Since the growth of prostate cancers *in vivo* can initially be restricted by anti-androgens, it would be of interest to determine whether p27 is up-regulated following androgen depletion of steroid-dependent prostate cancer.

Increasing data from studies of p27 *in vivo*, in human prostate cancers, suggest a role for this KIP in prostate epithelial cell growth. Several recent reports indicate a prognostic role for p27 in prostate cancer. Low or undetectable levels of p27 protein in primary prostate carcinoma have been shown to correlate with increased proliferative index (Guo *et al.*, 1997), increasing tumor grade (Tsihlias *et al.*, 1998; Yang *et al.*, 1998; Guo *et al.*, 1997; Cote *et al.*, 1998), shorter disease-free survival (Tsihlias *et al.*, 1998;

Yang *et al.*, 1998; Cote *et al.*, 1998; Cordon-Cardo *et al.*, 1998), and decreased overall survival (Cote *et al.*, 1998). In our study of the prognostic value of p27 in human prostate cancer, strong p27 staining was uniformly seen in benign prostatic epithelial components in all tumor sections. p27 staining was variable in prostatic intraepithelial neoplasia and reduced in most prostate cancers. A small subset of tumors, treated with pre-operative androgen ablation therapy prior to radical prostatectomy, tended to show higher expression of p27 protein than that in untreated cases. The few tumors that showed a persistence of low p27 staining (less than 25% of tumor nuclei positive) after androgen ablation therapy had the worst prognosis (Tsihlias *et al.*, 1998). In their study of p27 expression in prostate cancer, Cordon-Cardo *et al.* (1998) found low or absent p27 in androgen-independent metastatic prostate cancers. These authors also raised the possibility that mechanisms leading to accelerated p27 degradation may contribute to the development of metastases and/or progression of prostate cancer to the androgen-independent state. Taken together, both studies of p27 in prostate cancer cell lines and in primary tumors raise the hypothesis that changes in pathways that regulate p27 levels may contribute to tumor progression and to growth modulation of human prostate cancer cells in response to various androgenic stimuli.

## Materials and methods

### Cell culture

The fast growing strain of LNCaP, LNCaP-FGC (Berns *et al.*, 1986), and cell lines PC-3 and DU145 were purchased from ATCC. Cells were grown in RPMI 1640 culture media plus 5% FBS without phenol red. All experiments were performed using LNCaP passages 25 to 40. Cells were grown to 80% confluence in 10 cm tissue culture plates and split 1:6. Cell populations growing asynchronously were then treated with variable concentrations (0.1–100 nM) of 5α-dihydrotestosterone (Sigma Laboratories, St. Louis, MO, USA) or ethanol vehicle (0.1% and 1.0%) alone as a control. Cells were treated for up to 96 h, and the media changed every 48 h. As controls, the AR-negative prostate cancer lines PC-3 and DU145 were cultured in DMEM supplemented with 5% FBS without phenol red. Asynchronously growing PC-3 or DU145 cells were treated with 100 nM DHT for 48 h prior to recovery for flow cytometry and protein analysis.

### Determination of PSA secretion

Cells were cultured on 10 cm plates in 10 ml media. At 30% confluence, cells were washed twice with PBS and then cultured with fresh medium with or without addition of 100 nM DHT. Conditioned media were collected at 48 h and PSA concentration determined using the Hybritech assay.

### Immunohistochemistry

LNCaP cells were plated on poly-L-lysine coated glass slides and cultured for 24 h. The media was then supplemented with 100 nM DHT and cells cultured for a further 48 h. Cells were then washed in PBS, fixed in 4% paraformaldehyde containing 0.2% Triton X-100 for 10 min at room temperature. Slides were then blocked with 3% hydrogen peroxide in methanol followed by normal horse serum (10% solution) and then incubated overnight at 4°C with anti-p27 monoclonal antibody (Transduction Laboratories, Lexington,

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KY, USA) diluted 1:1000 (0.25 µg/ml) in PBS. Slides were then reacted with biotin-labeled anti-mouse IgG and incubated with preformed avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL, USA) was then added in the presence of horseradish peroxidase. Cells were photographed using a Wildervet microscope equipped with a Wild Leitz MPS 52 photocamera (Wild Leitz Ltd., Heerbrugg, Switzerland).

#### Flow cytometric analysis

Cells were pulse-labeled with 10 µM bromodeoxyuridine (BrdU) for 2 h at intervals after addition of DHT to asynchronously growing cells. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 95°C to expose labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton Dickinson) and counterstained with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

#### Immunoblotting

Cells were lysed in ice cold NP-40 lysis buffer (0.1% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis and 20–100 µg protein per lane resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer and blotting was as described (Dulic *et al.*, 1992). Proteins were detected by electrochemiluminescence (ECL). Densitometry was performed using the Molecular Dynamics Imaging system and ImageQuant software to quantitate the relative amounts of p27 protein detected on Western blots. For detection of cyclin E-associated proteins by immunoprecipitation-Western analysis (IP-Western), cyclin E was immunoprecipitated from 200 µg protein lysate, complexes resolved, blotted and blots reacted with cyclin E, cdk2, p21, and p27 antibodies. For detection of cdk4 complexes, 100–400 µg protein lysate was immunoprecipitated with cdk4 antibody and associated cyclin D1, p15, p16, p21, and p27 proteins detected by immunoblotting. For immunodepletion of p27, p27 was serially immunoprecipitated three times from 200 µg protein lysate and then cyclin E was immunoprecipitated from the p27-depleted lysate. The amounts of immunoprecipitable cyclin E protein, associated cdk2 and p27 proteins, and kinase activities prior to and after p27 immunodepletion were compared using IP-Western blotting and IP-kinase assays.

#### Oligonucleotide transfections

The sequences of the GSS422 antisense (ASp27) and mismatch p27 GS5585 (MSp27) C-5-propyne modified phosphorothioates used were 5'-TGGCTCTCXTGCGCC-3' and 5'-TGGCTCXCTTGCGCC-3', respectively. X indicates the proprietary 'G-clamp' modification of these oligonucleotides, provided by Gilead Sciences. Asynchronously growing cell cultures were transfected with oligonucleotides at a concentration of 50 nM using cytosectin G3815 at 2.5 µg/ml, as described (St.Croix *et al.*, 1996). After 6 h the transfection

cocktail was removed and replaced with complete medium containing 100 nM DHT. Cells were recovered for flow cytometric and protein analysis 36 h thereafter. 100 nM DHT-arrested cells were also transfected with ASp27, however, the reduction of p27 was considerably less in these cells than after transfection of ASp27 into asynchronously growing cells. The minor reduction in p27 protein levels in these DHT arrested cells was not sufficient to cause release from G0.

#### Cyclin-dependent kinase assays

Cdk4 kinase assays were performed using the method of Matsushime *et al.* (1994), using a truncated recombinant retinoblastoma protein as substrate. Quantitation of radioactivity incorporated in the substrate was performed using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin E-associated kinase assays were performed using either a monoclonal anti-cyclin E antibody (mAb E172, from E Harlow, Mass. General, MA, USA) or a polyclonal anti-cyclin E antibody (from D Agarwal, Lee Moffat Cancer Center, FL, USA). Histone H1 was used as substrate for cyclin E-associated kinase assays. In each case background activity was determined using a non-specific antibody as a control. Background activity was subtracted, and kinase activities were graphed as a per cent maximum activity.

#### Antibodies

The following antibodies were used in the immunoblotting experiments: pRB mouse mAb from Pharmingen; cdk2 rabbit pAb sc-163, cdk4 rabbit pAb sc-260, cdk6 rabbit pAb sc-172, cyclin D1 mouse mAb HD11, cyclin A rabbit pAb sc-596, cyclin B1 mouse mAb sc-245, and p21 rabbit pAb sc-397, all from Santa Cruz Biotechnology, CA, USA; p27 mouse monoclonal antibody from Transduction Laboratories, Lexington, KY, USA. PSA antibody was purchased from DAKO, Denmark. Monoclonal PSTAIRE antibody was a gift from S Reed (The Scripps Research Institute, CA, USA); cyclin D1 mouse mAb DCS-11 and p16 mouse mAb DCS-50, from J Bartek (Danish Cancer Society, Denmark); E12 and E172, mouse monoclonal antibodies to cyclin E from E Harlow (Mass. General, MA, USA); and cdk4 rabbit polyclonal antibody, for use in immunoprecipitation of cdk4, was provided by D Beach (CSH Labs, NY, USA). A monoclonal antibody, JC-6, which recognizes the third ankyrin repeat of human p16 (Enders *et al.*, 1995) and cross reacts with human p15, was used for immunoblotting of p15 in these studies. Results were confirmed by repeat biologic assays with different cell lysates.

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